



HALE AND DORR  
C O U N S E L L O R S   A T   L A W

60 STATE STREET, BOSTON, MASSACHUSETTS 02109  
617-526-6000 • FAX 617-526-5000

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27 September 1996

Assistant Commissioner for Patents  
**BOX APPLICATIONS**  
Washington, DC 20231

Re:    *Applicants:*            BENNETT *et al.*  
      *Serial No.:*            To Be Assigned  
      *For:*                    USE OF HEPARINASE TO DECREASE  
                                     INFLAMMATORY RESPONSES  
      *Filed:*                   27 September 1996  
      *Attorney Docket No.:* 104385.140

Sir:

Enclosed herewith for appropriate action by the United States Patent and Trademark Office are the following documents:

1.    New United States Patent application entitled:

**USE OF HEPARINASE TO DECREASE INFLAMMATORY RESPONSES**

and naming as the inventors:

*FOC*  
BENNETT, D. CLARK  
CAUCHON, ELIZABETH  
FINK, DOMINIQUE  
GROUX, BRIGETTE  
HSIA, ARIANE  
DANAGHER, PAMELA  
ZIMMERMAN, JOSEPH

**EXPRESS MAIL CERTIFICATION**

"Express Mail" mailing label number EM256700517US

Date of Deposit: 27 September 1996

I hereby certify that this correspondence and the enclosures referenced herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box APPLICATIONS, Washington, DC 20231.

*Karen Corey*  
Karen Corey

WASHINGTON, DC

BOSTON, MA

MANCHESTER, NH

The application comprising:

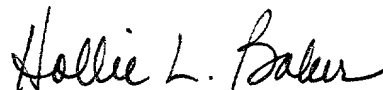
49 pages of Specification;  
2 pages of Claims;  
1 page Abstract; and  
19 sheets of informal drawings  
(depicting 19 Figures)

2. self-addressed, stamped postcard.

It is respectfully requested that the attached postage paid postcard be stamped with the unofficial serial number, and that it be returned to the undersigned as soon as possible.

This patent application is being submitted under 35 U.S.C. §111(a), claiming priority to United States Provisional Application 60/004,622, filed 29 September 1995, under 35 U.S.C. §119(e) and is submitted under 37 CFR §1.53(d) and 35 U.S.C. §111(a), without Declaration and without filing fee.

Respectfully submitted,



Hollie L. Baker  
Registration No. 31,321

HLB:KLC  
Enclosures

Hale and Dorr  
1455 Pennsylvania Avenue, NW  
Suite 1000  
Washington, DC 20004  
(202) 942-8400 telephone  
(202) 942-8484 facsimile

962600 692260



08/722659

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

BY  
D. CLARK BENNETT  
ELIZABETH CAUCHON  
DOMINIQUE FINK  
BRIGITTE GROUX  
ARIANE HSIA  
PAMELA DANAGHER  
AND  
JOSEPH ZIMMERMANN

FOR  
USE OF HEPARINASE TO DECREASE INFLAMMATORY RESPONSES

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## USE OF HEPARINASES TO DECREASE INFLAMMATORY RESPONSES

This application is related to U.S. provisional application serial no. 60/004,622, filed 29 September 1995, which is expressly incorporated herein by reference.

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### Field of the Invention

This invention is in the field of medical treatments and is directed to the use of heparinase enzyme as a treatment or prophylactic for reducing localized inflammatory responses.

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### Background of Invention

An inflammatory response is local response to cellular injury that is marked by capillary dilation, leukocytic infiltration, redness, heat, and pain and serves as a mechanism initiating the elimination of noxious agents and of damaged tissue.

A generalized inflammatory response within a tissue occurs by the recruitment of leukocytes to the tissue. Destruction of bacteria, foreign materials and/or damaged cells occurs through phagocytosis and/or extracellular degranulation (secretion of degradative enzymes, antimicrobial proteins and myeloperoxidase, which forms superoxides from secreted  $H_2O_2$ ). While most localized inflammatory responses are beneficial, harmful inflammatory responses can occur. Many harmful inflammatory responses also involve accumulation of leukocytes within a tissue. This accumulation results in the destruction of viable cells and tissue. In addition to damaging tissue, these responses are detrimental to, or debilitating for, the afflicted individual. Examples of detrimental inflammatory responses can include the following; ischemia/reperfusion injury following myocardial infarction, shock, stroke, organ transplantation, cardiopulmonary bypass surgery, allograft rejection, rheumatoid arthritis, antigen-induced asthma, allergic rhinitis, and glomerulonephritis (see the review in; Harlan, et al., *Immunol. Rev.*, 114:5-12, 1990; Carlos and Harlan, *Blood*, 84:2068-2101, 1994.)

35

Leukocyte recruitment involves a cascade of cellular events, beginning with activation of vascular endothelium by damaged or infected tissue adjacent to the

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endothelium. Activation of the endothelium results in enhanced adhesion of leukocytes to the endothelial cells, and transendothelial migration (extravasation) by bound leukocytes into the damaged tissue. Endothelial activation is manifested by a short-term, rapid, and/or a long-term stimulation of the endothelial cells.

Activators such as thrombin, chemoattractant leukotrienes,  $B_4$ ,  $C_4$  and  $D_4$  ( $LTB_4$ ,  $LT C_4$  &  $LT D_4$ ), and histamine cause rapid, transient (<30 minutes) endothelial cell activation, independent of protein synthesis, and can increase endothelial cell surface levels of the chemoattractants such as platelet activating factor (PAF; a glycerophospholipid) and  $LTB_4$  (shown for histamine), and adhesion molecules; ICAM-1 (shown for thrombin) and P-selectin (Zimmerman, et al., *J. Cell Biol.*, 110:529-540, 1990; Sugama, et al., *J. Cell Biol.*, 119:935-944, 1992; McIntyre, et al. *Proc. Natl. Acad. Sci. USA*, 83:2204-2208, 1986; Lorant, et al., *J. Cell Biol.*, 115:223-234, 1991). The outcome of rapid activation of endothelial cells is increased leukocyte adhesion to the endothelium (Hoover, et al., *Proc. Natl. Acad. Sci. USA*, 81:2191-2194, 1984; Zimmerman, et al., *J. Cell Biol.*, 110:529-540, 1990; Hanahan, et al. *Ann. Rev. Biochem.*, 55:483-509, 1986). However, increased  $LTB_4$  surface levels have not been shown to directly increase transendothelial migration of neutrophils (Hughes, et al., *Prost. Leuk. Essent. F. A.*, 45:113-119, 1992), and in certain situations, PAF is not necessary for adhesion of leukocytes to activated endothelium (Kuijpers, et al., *J. Cell Biol.*, 117:565-574, 1992).

Long-term (hours in duration) protein synthesis dependent, endothelial cells activation is produced by cytokines, such as IL-1b and TNF-a, and by lipopolysaccharide (LPS) and results in maintenance of increased surface levels of adhesion molecules: E-selectin, P-selectin, ICAM-1 and VCAM-1 (reviewed by Carlos, and Harlan, *Blood*, 84:2068-2101, 1994). IL-1b and TNF-a also increase the synthesis of PAF by endothelial cells (Kuijpers, et al., *J. Cell Biol.*, 117:565-574, 1992). In addition, endothelial cell activation by IL-1b, TNF-a, LPS and histamine has been shown to increase the synthesis and secretion of the chemokine, IL-8 (Strieter, et al., *Science*, 243:1467-1469, 1989; Jeannin, et al., *Blood*, 84:2229-2233, 1994).

Chemokines, IL-8 and MCP-1, have been shown to be produced by and to be present on the endothelial cells surface (Huber, et al., *Science*, 254:99-102, 1991; Springer, *Nature*, 346:425-434, 1990). The chemokine, MIP-1b, has been shown to be present on lymph node endothelium, *in vivo* (Taub, et al., *Science*, 260:355-359, 1993; Tanaka, et al., *Nature*, 361:79-82, 1993). The chemokines; RANTES, MIP-a, MIP-b, MCP-1 and IL-8 are all heparin binding proteins, which after being secreted, bind to cell surface and extracellular matrix proteoglycans possessing heparin and heparan sulfate moieties (reviewed by Miller, et al., *Crit. Rev. Immunol.*, 12:17-46, 1992).

Heparin and heparan sulfate are similar glycosaminoglycan moieties found interspersed on the same unbranched carbohydrate chains. They are covalently attached to the protein backbones of proteoglycans. Despite what these two names imply, heparin is more highly sulfated than is heparan sulfate. Proteoglycans are present on cell surfaces and in extracellular matrices (e.g. in the basement membrane of endothelium). Because of difficulty in distinguishing regions of heparin and heparan sulfate on the same carbohydrate chain, little data exists on the binding preference of chemokines for either heparin or heparan sulfate moieties. There is some indication that chemokines IL-8 and GRO bind with greater affinity to heparan sulfate than heparin, and that PF4 and NAP-2 bind with greater affinity to heparin moieties (Witt, and Lander, *Curr. Biol.*, 4:394-400, 1994). Generally, chemokines are referred to as heparin binding proteins. C-terminal regions of the chemokines IL-8, PF4, MCP-1 and NAP-2 have been shown to form an  $\alpha$ -helix, and to bind to heparin/heparan sulfate (Webb, et al., *Proc. Natl. Acad. Sci. USA*, 90:7158-7162, 1993; Zucker, et al., *Proc. Natl. Acad. Sci. USA*, 86:7571-7574, 1989; Matsushima, et al., in *Interleukins: Molec. Biol. Immunol.*, ed. Kistimoto, Karger, Basel, 236-265, 1992). This is likely to be a structure, common to all of the chemokines.

All of the molecules mentioned above, which are expressed by activated endothelial cells (PAF, LTB<sub>4</sub>, selectins, CAMs and chemokines), are present on the endothelial cell surface, and are localized to vascular endothelium adjacent to sites of damaged tissue. Blood-borne leukocytes which interact with these molecules will also be localized in their binding in the area of the damaged tissue. The

outcome of long-term activation of endothelium is increased adhesion and extravasation of leukocytes and a significant localized accumulation of leukocytes in adjacent tissue, which cannot occur during short-term activation (Ebisawa, et al., *J. Immunol.*, 149:4021-4028, 1992; Huber, and Weiss, *J. Clin. Invest.*, 83:1122-1129, 1989; Oppenheimer-Marks, et al., *J. Immunol.*, 145:140-148, 1990).

Adhesion of leukocytes to endothelium is thought to be a two step process (reviewed by Carlos, and Harlan, *Blood*, 84:2068-2101, 1994). Initially, leukocytes roll along the surface of blood vessels. Increased rolling is initially mediated on vascular endothelium (within the first 30 minutes) by interactions between Sialyl Lewisx structures on the leukocyte surface and P-selectin and E-selectin, which are increased on activated endothelial cells (Ley, et al., *Blood*, 85:3727-3735, 1995). Increased rolling is also mediated (after 40 minutes) by interactions between L-selectin on leukocyte cellular membranes and heparin-like molecules on the vascular endothelium, which are cytokine-inducable (Karin. et al., *Science*, 261:480-483, 1993), or between L-selectin on lymphocytes and vascular addressins; GlyCAM-1, CD34 and MAdCAM-1 on high endothelial venules (HEVs) in lymphoid tissue. The second step, firm adhesion of leukocytes to endothelial cells, is based on interactions between leukocyte integrins (e.g. LFA-1, Mac-1, VLA-4) and endothelial cellular adhesion molecules (CAMs; e.g. ICAM-1, ICAM-2, VCAM-1, MAdCAM-1). Leukocytes flatten on the endothelial surface, and shed L-selectin, concomitant with firm adhesion (Kishimoto, et al., *Science*, 245:1238-1242, 1989; Jutila, et al., *J. Immunol.*, 143:3318-3324, 1989; Smith, et al., *Clin. Invest.*, 87:609-618, 1991).

Selectin and CAM levels increase on the endothelium surface in response to many cytokines and chemoattractants. These increases are dependent on synthesis and/or secretion of additional selectin and CAM molecules onto the cell surface. In contrast, activation of leukocytes for firm adhesion has been shown to occur within seconds (Bargatze, et al., *J. Exp. Med.*, 178:367-373, 1993), through increased secretion of integrins, and more importantly, through induction of conformational changes in cell surface integrins (integrin activation), which permits tight binding of the integrins to CAMs (reviewed in Zimmerman, *Immunol. Today*, 13:93-99, 1992).

PAF and E-selectin can activate integrins for endothelial cell adhesion (Lorant et al., *J. Biol. Chem.*, 115:223-234,1991; Lo, *J. Exp. Med.*, 173:1493-1500,1991).

5 The presence of MIP-1b, immobilized by binding to CD44 (possesses heparin/heparan sulfate moieties), or a heparin-BSA conjugate, has been shown to be required for CD8+ T-cell binding to immobilized VCAM-1 molecules. This binding was shown to be blocked by an antibody to VLA-4, indicating that MIP-1b activates VLA-4 on the T-cell surface (Tanaka, et al., *Nature*, 361:79-82, 10 1993). An increase in the level of integrin, CD18 (part of Mac-1), on the surface of neutrophils has been shown to occur when neutrophils contact endothelium, which has been stimulated with IL-1b. An antibody to IL-8 inhibited the CD18 up-regulation, and also inhibited neutrophil adhesion (Huber, *Science*, 254:99-102, 15 1991). Thus, chemokines can act as direct activators of leukocyte adhesion. In contrast, Luscinskas et al. (*J. Immunol.*, 149:2163-2171, 1992) has demonstrated that pretreatment of neutrophils with IL-8 inhibits neutrophil attachment, and addition of exogenous IL-8 detached neutrophils adhering to activated endothelial cells. Rot (*Immunol. Today*, 13:291-294, 1992) has reconciled these contradictory 20 results by proposing that IL-8 bound to the endothelial cells surface promotes adhesion, while soluble IL-8 can inhibit it.

Different chemokines activate different leukocytes. IL-8 activates neutrophils, eosinophils and T cells. RANTES activates monocytes, eosinophils and T cells. MCP-1 activates monocytes. MIP-1a activates CD4+ T cells, monocytes 25 and B cells, while MIP-1b activates monocytes and CD8+ T cells (reviewed in Lasky, *Current Biol.*, 3:366-378, 1993). Different combinations of selectins, integrins, CAMs and chemokines are thought to select for the adhesion and migration of the leukocyte subtypes observed in different inflamed tissues (Butcher, *Cell*, 67:1033-1039, 1991).

30 The importance of interactions of integrins, CD11/CD18 (Mac-1), and ICAMs in adhesion and extravasation of leukocytes has been demonstrated in numerous systems by the use of antibodies to these molecules. The antibodies interfere with the function of the adhesion molecules and block or reduce leukocyte recruitment. The leukocyte adhesion deficiency (LAD) Type I syndrome 35 results in a partial or total absence of the integrin, CD18, on the leukocytes of



affected patients. Neutrophil recruitment to sites of inflammation is negligible. However, monocyte and eosinophil recruitment is normal, indicating that an  
5 alternative set of adhesion molecules may function for recruitment of these cells, perhaps VLA-4 and VCAM-1 (Harlan, *Clin. Immunol. Immunopath.*, 67:S16-S24, 1993). VLA-4 is not expressed by neutrophils (Winn and Harlan, *J. Clin. Invest.*, 92:1168-1174, 1993). As mentioned previously, chemokines are important for  
10 activation and increased surface levels of integrins VLA-4 and CD18 on leukocytes. IL-8 immobilized on a polycarbonate filter has been shown to be adequate for directing migration of neutrophils through the filter (Rot, *Immunol. Today*, 13:291-294). Huber, et al. (*Science*, 254:99-102, 1991) has shown that a  
15 transendothelial gradient of bound IL-8, produced by IL-1b stimulated endothelial cell monolayers, is necessary for extravasation of neutrophils. These neutrophils were pre-activated with IL-8 and did bind to the endothelial cells, but did not migrate until the IL-8 gradient was present. This gradient extended from the  
20 endothelial cells luminal surface through the basement membrane underlying the endothelial cell monolayer. Washing bound IL-8 from the basement membrane underlying activated endothelial cells prevented migration across the monolayer. In addition, an antibody to IL-8 inhibited 70-80% of the neutrophil migration. Kuijpers et al. (*J. Cell Biol.*, 117:565-572, 1992) used an anti-IL-8 antibody to  
25 produce a 60% reduction in neutrophil migration across IL-1b and TNF-a activated endothelium, and addition of a PAF receptor antagonist produced an 85-90% reduction in migration. These results are in contrast to experiments which showed that IL-8 pretreated neutrophils were inhibited in their ability to migrate through an activated endothelial cell monolayer (Luscinskas et al., *J. Immunol.*, 149:2163-2171, 1992). Thus, it is likely that chemokines not only activate  
30 leukocytes for adhesion, but that a bound gradient of chemokine is important in extravasation of leukocytes. The presence of soluble chemokine can interfere with adhesion and migration along a bound chemokine gradient. As discussed below, *in vivo* localized concentration increases in soluble chemokines would be minimized by blood flow.

35 Once activated leukocytes have begun to accumulate within a damaged tissue, they can augment the accumulation of additional leukocytes, by synthesis

and secretion of cytokines, chemokines, and LTB<sub>4</sub>. LPS has been shown to directly increase monocyte IL-1b expression (Porat, et al., *FASEB J.*, 6:2482-2489, 1992).

5 IL-8, IL-1b and TNF-a are produced by neutrophils activated with GM-CSF, another cytokine produced by activated macrophages, endothelium and T-cells (McCain, et al., *Am. J. Respir. Cell Molec. Biol.*, 8:28-34, 1993; Lindemann, et al., *J. Immunol.* 140:837-839, 1988; Lindemann, et al., *J. Clin. Invest.*, 83:1308-1312, 1989). IL-1b and TNF-a have been shown to stimulate monocytes, thereby

10 increasing the expression of the chemokines, IL-8 and MIP-1a (Lukacs, et al., *Blood*, 82:3668-3674, 1993). Activated neutrophils and monocytes have been shown to be a major source of LTB<sub>4</sub> production (Samuelsson, et al., *Science*, 237:1171-1176, 1987; Brach, et al., *Eur. J. Immunol.*, 22:2705-2711, 1992). As discussed previously, LTB<sub>4</sub>

15 is not directly involved in further recruitment of leukocytes, but because neutrophils stimulated with LTB<sub>4</sub> produce IL-8, the LTB<sub>4</sub>-stimulated neutrophils could promote further neutrophil recruitment, indirectly, through formation of an IL-8 gradient (McCain, et al., *Am. J. Respir. Cell Molec. Biol.*, 10:651-657, 1994). The continued recruitment of leukocytes by these leukocyte-derived activators would

20 require using the vascular endothelium as an intermediate. Endothelial cells and basement membranes would bind and display neutrophil-derived chemokines, forming a gradient, or leukocyte-derived cytokines would activate the endothelium, which would also cause the creation of a chemokine gradient.

Blood flow in the vasculature would prevent a localized concentration

25 increase in soluble activation factors (cytokines, chemokines and chemoattractants), produced by a tissue-localized inflammatory response. If a local inflammation is producing high blood concentrations of activators, a systemic activation of leukocytes could occur (Finn, et al., *J. Thorac. J. Surg.*, 105:234-241, 1993). The activated leukocytes would then bind transiently to

30 unactivated endothelium and/or degranulate, causing sepsis (Sawyer, et al., *Rev. Infect. Dis.*, 11:S1532-1544, 1989). In situations where some blood-borne leukocytes are activated by a localized inflammation (not all of the activated leukocytes extravasate), the activated leukocytes would produce and secrete additional

35 cytokines, chemokines, and LTB<sub>4</sub> into the blood. This increase in activator

concentration could up-regulate unactivated cells and amplify the systemic response.

Although the mechanism of inflammatory responses has been given in some detail, there is still a need for an effective treatment and pharmaceutical compositions for reducing or preventing localized inflammatory responses.

### Summary of Invention

This invention is directed to the discovery that heparinase degrading enzymes, either separately or in combination, can be used to decrease localized inflammatory response. The heparinases useful in this invention can be from a variety of sources: heparinases I, II, and III from the Gram negative bacterium *Flavobacterium heparinum*, heparinase from *Bacteroides* strains, heparinase from *Flavobacterium* Hp206, heparinase from *Cytophagia* species, and heparanases from mammalian cells. These enzymes, either singly or in combination, are referred to herein as heparinase or heparinase enzyme.

Heparin and heparan sulfate moieties are degraded on the surface of endothelial cells and from basement membranes by administration of heparinase. Removal of heparin and heparan sulfate moieties from up-regulated proteoglycans on activated endothelial cells prevents L-selectin, found on leukocytes, from interacting with the proteoglycans. By decreasing L-selectin-proteoglycan interactions, leukocyte rolling on activated endothelium can be inhibited.

In addition, when heparin and heparan sulfate moieties are removed from the surface of activated endothelial cells and from their basement membrane, chemokines, which are bound to the heparin and heparan sulfate, are released from the cell surfaces and basement membrane. The loss of bound chemokines decreases the localized concentration of chemokines and disrupts the chemokine gradient produced by activated endothelium, thereby inhibiting chemokine activation of rolling leukocytes, which is required for firm adhesion, and preventing extravasation of leukocytes along the chemokine gradient. By this invention, decreased leukocyte rolling, activation and extravasation can inhibit localized tissue inflammatory responses by interfering with fundamental mechanisms of leukocyte recruitment.

Heparinase enzyme can be targeted to specific cell types, tissues or organs by the selected method of administration, which deliver localized high concentrations of the enzymes or physically limit the dispersal of the enzymes. Additionally, according to this invention heparinase can be targeted by fusion of the enzymes to binding domains from antibodies, growth factors or adhesion molecules. The fusion proteins are produced by construction and expression of gene fusions in recombinant organisms. As examples, the binding domains can recognize cell surface molecules on activated endothelium (e.g., ICAM-1, VCAM-1, P-selectin, E-selectin), or on endothelial cell subtypes (e.g., GlyCAM-1). Targeted fusion enzymes can increase the number and specificity of indications, while decreasing the amounts of enzyme required for efficacy and possible side-effects resulting from treatments.

### Brief Descriptions of the Drawings

Figure 1 is a graph of the counts of <sup>35</sup>S-heparin/heparan sulfate released from the surface of endothelial cells by 1.0 IU/ml of heparinase III, which were separated according to size on a gel filtration column. The diamonds indicate counts released by a 5 minute digest, the squares indicate counts released by a 30 minute digest, and the triangles indicate counts released by a 60 minute digest. Background counts from fractionation of mock digests have been subtracted from the fractions derived from the heparinase III digests.

Figure 2A and 2B are graphs of the percent of heparin/heparan sulfate present on the unactivated (2A) and IL-1b activated (2B) human endothelial cell line at the indicated times after treatment with 0.1 IU/ml heparinase I, II or III for 1 hour. <sup>125</sup>I-bFGF binding to cell surface heparin was used to determine the amount of heparin/heparan sulfate present. Results for heparinase I, II or III treated cells are indicated by diamonds, squares or triangles, respectively. The vertical lines indicate the standard error of the means.

Figure 3A and 3B are graphs of the percent of heparin/heparan sulfate present on the unactivated (3A) and IL-1b activated (3B) human endothelial cell line at the indicated times after treatment with 1.0 IU/ml heparinase I. <sup>125</sup>I-bFGF binding to cell surface heparin was used to determine the amount of heparin/heparan sulfate present. Results for 1, 3 or 5 hour treated cells are

indicated by diamonds, squares or triangles, respectively. The vertical lines indicate the standard error of the means.

5 Figure 4A and 4B are graphs of the percent of heparin/heparan sulfate present on the unactivated (4A) and IL-1b activated (4B) human endothelial cell line at the indicated times after treatment with 1.0 IU/ml heparinase III. 125I-bFGF binding to cell surface heparin was used to determine the amount of heparin/heparan sulfate present. Results for 1, 3 or 5 hour treated cells are  
10 indicated by diamonds, squares or triangles, respectively. The vertical lines indicate the standard error of the means.

Figure 5 contains graphs displaying the levels of IL-8 released from IL-1b activated human endothelial cell layers by treatment with 1.0 IU/ml of heparinases; I (5A), II (5B), I+III (bars containing diagonal lines; 5B), and III (5C).  
15 The bars represent the percent difference in the concentration of IL-8 found in; supernatants from activated endothelial layers treated with heparinases, versus untreated supernatants from activated endothelial layers (containing only secreted IL-8). Standard errors for these percentage differences are indicated by vertical  
20 lines. The lines overlaid on the bars indicate the concentration of IL-8 in the supernatants from the heparinase treated cell layers. The standard errors of these measurements are also indicated by vertical lines (not always visible).

Figure 6 is a graph of the level of neutrophil adhesion to endothelial cells, which were unactivated, IL-1b activated, or treated with 0.1 IU/ml of heparinases  
25 I, II or III after IL-1b activation. The level of adhesion is expressed as the percent of added neutrophils, which are adhering.

Figures 7A, 7B and 7C are graphs of the percent inhibition of neutrophil extravasation through IL-1b activated endothelial cell layers, which were treated with heparinases I, II or III, respectively. The bars containing diagonal lines  
30 represent results of one hour treatments with 1.0 IU/ml of heparinase. The white bars represent results of one hour treatments with 0.1 IU/ml of heparinase. The black bars represent results of 15 minute treatments with 0.1 IU/ml of heparinase I or III, and the bar containing vertical lines represents results of 15 minute  
35 treatments with 1.0 IU/ml of heparinase II. The standard deviations for the percent inhibitions are indicated by vertical lines. The small asterisks indicate

results of one hour treatments that were significantly different from the results of the 15 minute treatment with the same heparinase ( $P < 0.05$ ). The large asterisks indicate the results of one hour treatments with 1.0 IU/ml of heparinase that were significantly different from the results of one hour treatments with 0.1 IU/ml of the same heparinase. The numbers in parentheses under the bars indicate the number of experiments included in each data set.

Figure 8 is a graph showing the activity of human heparinase (b-thromboglobulin) on ECM at pH 5.8 and 7. The solid bars represent the percent difference in  $^{35}\text{SO}_4$  released from ECM treated with 1 ug of human heparinase versus that released from untreated ECM. The bars containing diagonal lines represent the percent difference in  $^{35}\text{SO}_4$  released from ECM treated with 5 ug of human heparinase versus that released from untreated ECM. The standard deviation of the means are indicated by vertical lines.

Figure 9 is a graph which displays the change in the level of neutrophil extravasation upon activation of HUVEC layers with IL-1b, and after treatment of activated HUVEC layers with human heparinase (hhep). The standard deviation of the means are indicated by vertical lines.

Figure 10 is a graph of rat plasma heparinase III concentrations over a five hour infusion period. Time points in the protocol are indicated by the arrows, with descriptions above the arrows. The vertical lines indicate the standard error of the means.

Figure 11 is a graph of the level of leukocyte rolling in the rat microvasculature after 3 hours of ischemia, during reperfusion. The circles indicate the levels in naive rats, the squares indicate the levels in sham treated rats which underwent ischemia, and the triangles indicate the levels in heparinase treated rats which underwent ischemia. The vertical lines indicate the standard error of the means.

Figure 12 is a graph of the level of leukocyte adhesion in the rat microvasculature after 3 hours of ischemia, during reperfusion. The circles indicate the levels in naive rats, the squares indicate the levels in sham treated rats which underwent ischemia, and the triangles indicate the levels in heparinase

treated rats which underwent ischemia. The vertical lines indicate the standard error of the means.

5 Figure 13 is a graph of the level of leukocyte extravasation in the rat microvasculature after 3 hours of ischemia, during reperfusion. The circles indicate the levels in heparinase treated rats which underwent ischemia. The vertical lines indicate the standard error of the means.

10 Figure 14 is a graph of the level of leukocyte extravasation in the rat microvasculature after 2 hours of ischemia, during reperfusion. The open bars are the percent difference in the levels in sham treated rats versus the levels in naive rats. The bars containing diagonal lines are the percent difference in the levels in heparinase treated rats versus the levels in naive rats. The vertical lines indicate  
15 the standard error of the means.

Figure 15 is a graph of the level of perfusion in rat postcapillary venules after 3 hours of ischemia, during reperfusion. The circles indicate the levels in naive rats, the squares indicate the levels in sham treated rats which underwent ischemia, and the triangles indicate the levels in heparinase treated rats which  
20 underwent ischemia. The vertical lines indicate the standard error of the means.

Figure 16 is a graph of the heart rate-blood pressure product for rabbits during ischemia and reperfusion with or without heparinase treatment. The open circles and squares are data for saline pretreated and reperfusion treated rats, respectively. The open pyramids and solid circles are data for heparinase  
25 pretreated and reperfusion treated rabbits, respectively (25 ug/ml target plasma levels for heparinase III). The solid squares, pyramids and diamonds are data for heparinase reperfusion treated rabbits with 5, 1.25 and 0.25 ug/ml target plasma levels of heparinase III, respectively. BASE indicates baseline levels. 30I indicates the level at 30 minutes of ischemia. 30R, 60R, 120R and 180R indicates the levels  
30 at 30, 60 120 and 180 minutes of reperfusion. The vertical lines indicate the standard deviation of the means.

Figure 17 is a graph of the percent of the infarct size vs. risk zone after ischemia and reperfusion in rabbit hearts, which underwent different heparinase  
35 treatments. The solid circles indicate the average levels for each treatment group. The open shapes indicate the levels for individual rabbits. CPT and CRT indicate

saline pretreated and reperfusion treated rabbits, respectively. DPT and DRT indicate heparinase pretreated and reperfusion treated rabbits, respectively. The numbers below DPT and DRT indicate the target level of heparinase III in the plasma (in ug/ml). The vertical lines indicate the standard deviation of the means.

Figure 18 is a graph of the concentration of heparinase III which was infused into the heparinase treated rabbits (in IU/ml). DPT and DRT indicate heparinase pretreated and reperfusion treated rabbits, respectively. The numbers below DPT and DRT indicate the target level of heparinase III in the plasma (in ug/ml). Con indicates control rabbits infused with saline. The vertical lines indicate the standard deviation of the means.

Figure 19 is a graph of the concentrations of heparinase III measured in the rabbit plasma during pretreatment and reperfusion. The circles indicate the actual concentrations measured in heparinase pretreated rabbits targeted for 25 ug/ml plasma concentrations of heparinase III. The squares, pyramids, triangles and diamonds indicate the actual concentrations measured in heparinase reperfusion treated rabbits targeted for 25, 5, 1.25 and 0.25 ug/ml plasma concentrations of heparinase III, respectively. BASE indicates baseline concentrations. 30P and 60P indicate concentrations at 30 and 60 minutes of pretreatment. 15R, 30R, 60R, 120R and 180R indicate concentrations at 15, 30, 60 120 and 180 minutes of reperfusion, respectively. The vertical lines indicate the standard deviation of the means.

#### **Detailed Description of the Invention**

Interactions between leukocytes and endothelium are critical to the progress of localized inflammatory responses. These critical interactions include functional contacts between endothelium bound chemokines and leukocyte chemokine receptors, and between leukocyte L-selectin and heparin/heparan sulfate proteoglycans on endothelium. This invention is based on the discovery and is directed to the use of heparinase enzyme and heparinase fusion protein to decrease leukocyte-chemokine and leukocyte-endothelial cells proteoglycan interactions, and thereby inhibiting localized inflammation.

Heparin and heparan sulfate are glycosaminoglycan moieties of proteoglycans located on the surface of many different cell types and also found



in the extracellular matrices produced by many cells. Endothelial cells produce extracellular matrix, primarily on their abluminal side, referred to as basement  
5 membrane. Endothelial cells, activated by certain cytokines or by other inflammatory response stimulators, increase their surface levels of heparin and heparan sulfate proteoglycans (excluding high endothelial venules), which act as inflammatory adhesion molecules, and interact with L-selectin on rolling  
10 leukocytes. This interaction increases contacts of leukocytes with the endothelium (increased rolling of leukocytes), which are necessary for subsequent steps in leukocyte recruitment. Activated endothelium also increases its synthesis and secretion of chemokines. The secretion of chemokines in turn increases the localized concentration of the same chemokines, because the chemokines bind to  
15 the heparin and heparan sulfate moieties of proteoglycans on the endothelial cells surfaces and in the basement membranes. This localized concentration gradient is required for activation of leukocytes for firm adhesion and extravasation, and results in leukocyte recruitment to inflammatory sites.

Heparinase enzymes have been found in microorganisms including  
20 *Flavobacterium heparinum* (Lohse and Linhardt, *J. Biol. Chem.* 267:2437-24355, 1992), *Bacteroides* strains (Saylers, et al., *Appl. Environ. Microbiol.* 33:319-322, 1977; Nakamura, et al., *J. Clin. Microbiol.* 26:1070-1071, 1988), *Flavobacterium* Hp206 (Yoshida, et al., 10th Annual Symposium of Glycoconjugates, Jerusalem 1989) and *Cytophagia* species (Bohn, et al., *Drug Res.* 41(I), Nr. 4:456-460, 1991). Heparanases  
25 from mammalian cells have also been described (Fuks, et al., US patent number 5,362,641, 1994; Hoogewerf et al., *J. Biol. Chem.* 270:3268-3277, 1995). The heparinases from *Flavobacterium heparinum*, heparinase I (EC 4.2.2.7), and heparinase II degrade heparin, while heparinase II also degrades heparan sulfate, as does heparinase III (EC 4.2.2.8). The products of complete digestion by these  
30 enzymes are mainly disaccharides, though small quantities of tetrasaccharides and oligosaccharides may persist. These enzymes can be used to remove cell surface and basement membrane glycosaminoglycans, heparin and heparan sulfate.

The removal of heparin and heparan sulfate from endothelial cells interferes  
35 with L-selectin interactions with endothelium, preventing increased leukocyte rolling. The removal of glycosaminoglycans from endothelial cells and basement

membranes also removes glycosaminoglycan bound chemokines, which are critical for leukocyte recruitment. Loss of endothelial cells bound chemokines decreases activation of leukocyte integrins and inhibits firm adhesion by the leukocytes. It also inhibits extravasation of leukocytes, because the leukocytes require the presence of a bound gradient of chemokine for transmigration. It is believed, without being limited, that unbound chemoattractants are depleted from the endothelium layer by blood flow, preventing formation of a significant soluble chemoattractant gradient.

Generally, after a one hour heparinase treatment, 50% of the digested cell surface and basement membrane heparin and heparan sulfate are replaced within 2 to 4 hours, and it is completely replaced within 12 to 16 hours. Longer treatment times (3 and 5 hours) greatly extended the time needed to replace the same amount of heparin/heparan sulfate. Inflammatory responses would be significantly diminished by a slow rate of replacement of cell surface heparin/heparan sulfate. Appropriate administration of heparinase could extend the duration of diminished inflammatory response.

#### Preparation of Heparinase

Individual heparinases or a combination thereof, that may be used in this invention can be prepared from a variety of sources. Heparinase may be prepared by isolation from bacterial or mammalian cells, either those which naturally produce the enzymes or have been genetically engineered to produce the enzymes as described in by known methods. In addition, mammalian heparanases from human cells may be isolated according to procedures for purification described by Fuks, et al. (U.S. 5,362,641, 1994).

#### Isolation of Heparinases from *Flavobacterium heparinum*

Heparinase enzymes can be purified from cultures of *Flavobacterium heparinum* as follows. *F. heparinum* is cultured in 15 L computer controlled fermenters, in a variation of the defined nutrient medium described by Galliher et al., *Appl. Environ. Microbiol.* 41(2):360-365, 1981. For fermentations designed to produce heparin lyases, semi-purified heparin (Celsus Laboratories) is included in the media at a concentration of 1.0 g/L as the inducer of heparinase synthesis. The cells are harvested by centrifugation and the desired enzymes released from

the periplasmic space by a variation of the osmotic shock procedure described by U.S. Patent No. 5,169,772 to Zimmermann, et al. (1992).

5 Proteins from the crude osmolate are adsorbed onto cation exchange resin (CBX, J.T. Baker) at a conductivity of between one and seven mhos. Unbound proteins from the extract are discarded and the resin packed into a chromatography column (5.0 cm i.d. x 100 cm). The bound proteins elute at a linear flow rate of 3.75 cm•min<sup>-1</sup> with step gradients of 0.01 M phosphate, 0.01  
10 M phosphate/0.1 M sodium chloride, 0.01 M phosphate/0.25 M sodium chloride and 0.01 M phosphate/ 1.0 M. sodium chloride, all at pH, 7.0 ± 0.1. Heparinase II elutes in the 0.1 M NaCl fraction while heparinases, I and III, elute in the 0.25 M fraction. Alternately, the 0.1 M sodium chloride step is eliminated and the three heparinases co- eluted with 0.25 M sodium chloride. The heparinase  
15 fractions are loaded directly onto a column containing cellulose sulfate (5.0 cm i.d. x 30 cm, Amicon) and eluted at a linear flow rate of 2.50 cm•min<sup>-1</sup> with step gradients of 0.01 M phosphate, 0.01 M phosphate/0.2 M sodium chloride, 0.01 M phosphate/0.4 M sodium chloride and 0.01 M phosphate/ 1.0 M. sodium chloride,  
20 all at pH, 7.0 ± 0.1. Heparinases II and III elute in the 0.2 M sodium chloride fraction while heparinase I elutes in the 0.4 M fraction. The 0.2 M sodium chloride fraction from the cellulose sulfate column is diluted with 0.01 M sodium phosphate to give a conductance less than 5 mhos. The solution is further purified by loading the material onto a hydroxyapatite column (2.6 cm i.d. x 20  
25 cm) and eluting the bound protein at a linear flow rate of 1.0 cm•min<sup>-1</sup> with step gradients of 0.01 M phosphate, 0.01 M phosphate/0.35 M sodium chloride, 0.01 M phosphate/0.45 M sodium chloride, 0.01 M phosphate/0.65 M sodium chloride and 0.01 M phosphate/ 1.0 M. sodium chloride, all at pH, 7.0 ± 0.1. Heparinase II elutes in a single protein peak in the 0.45 M sodium chloride fraction while  
30 heparinase III elutes in a single protein peak in the 0.65 M sodium chloride fraction. Heparinase I is further purified by loading material from the cellulose sulfate column, diluted to a conductivity less than 5 mhos, onto a hydroxyapatite column (2.6 cm i.d. x 20 cm) and eluting the bound protein at a linear flow rate of 1.0 cm•min<sup>-1</sup> with a linear gradient of phosphate (0.01 to 0.25 M) and sodium  
35

chloride (0.0 to 0.5 M). Heparinase I elutes in a single protein peak approximately mid-way through the gradient.

5 The heparinase enzymes obtained by this method are greater than 98.5% pure as estimated by reverse phase HPLC analysis (BioCad, POROS II). Purification results for the heparinase enzymes are shown in Table A.

10 TABLE A: Purification of heparinase enzymes from  
*Flavobacterium heparinum* fermentations

		activity	specific activity	yield
	sample	(IU)	(IU/mg)	(%)
15	fermentation			
	heparin degrading	94,500		100
	heparan sulfate			
	degrading	75,400	ND	100
	osmolate			
20	heparin degrading	52,100		55
	heparan sulfate			
	degrading	42,000	ND	56
	cation exchange			
25	heparin degrading	22,600		24
	heparan sulfate			
	degrading	27,540	ND	37
	cellufine sulfate			
	heparin degrading	19,200		20
30	heparan sulfate			
	degrading	9,328	30.8	12
	hydroxylapatite			
	heparinase 1	16,300	115.3	17
	heparinase 2	2,049	28.4	3
35	heparinase 3	5,150	44.5	7

### Isolation of Recombinant Enzymes

Glycosaminoglycan degrading enzymes also can be isolated from recombinant expression systems such as the heparinase I expression system described by Sasisekharan, et al., Proc. Natl. Acad. Sci. USA 90:8660-8664, 1993; the heparinase, II and III, expression systems described in co-pending U.S. Patent application serial No. 08/258,639, "Nucleic Acid Sequences and Expression Systems for Heparinase II and Heparinase III Derived From *Flavobacterium heparinum*" by Su, et al., filed June 10, 1994, the teachings of which are incorporated herein. In these expression systems, the *F. heparinum* genes are isolated and cloned into plasmids downstream from an inducible promoter. The plasmids are introduced into *E. coli* and the expression of the desired enzyme directed by a suitable induction method such as temperature shift or addition of IPTG to the medium.

The enzymes can be recovered in a purified form by a modification of the methods described herein. Cell disruption is achieved by homogenization, sonication or enzymatic treatment to break the cell wall and release cytoplasmic components. If enzyme synthesis results in aggregation, the aggregate can then be dissolved by a denaturing agent, 3 to 6 M guanidine HCl or 4 to 8 M urea and the protein refolded by removal of the denaturing agent through dialysis or dilution. The refolded enzyme can be further purified using the liquid chromatographic methods described above.

### Construction of Fusion Proteins

Fusion proteins incorporating heparinase enzyme fused to proteins with specific binding properties can be created by recombinant molecular biology techniques. By choosing an appropriate binding protein, heparinase activity can be targeted to specific sites, *in vivo*. ICAM-1 has been shown to be preferentially expressed on the surface of activated endothelial cells (Dustin, et al., J. Immunol., 137:245-254, 1986). As examples of fusion proteins; an antibody, Fab fragment or variable region, specific for ICAM-1, VCAM-1 or P-selectin, when fused to heparinase enzyme or an active portion thereof, localizes heparinase activity near the luminal and abluminal surfaces of activated endothelium. Heparin and heparan sulfate moieties are removed in this area, causing breakdown of the

chemokine gradient produced by the endothelium. As other examples, fusion of heparinase enzyme, or an active portion thereof, to the I-domain of LFA-1 or Mac-1 (both bind to ICAM-1) targets activated endothelium for removal of heparin and heparan sulfate, inhibiting leukocyte rolling and chemokine gradient formation. Receptors for cytokines such as IL-1b, are up-regulated on activated endothelium and provide another target for binding of fusion proteins. By fusing IL-1b, or the receptor binding domain of IL-1b to heparinase targeting can also be achieved. The fusion proteins can decrease inflammatory responses at lower blood concentrations than is required for comparable decreases using unfused heparinase. In addition, other cells in the vascular system will be less affected by the enzyme activity of the fusion protein, reducing possible side effects of treatments.

Heparinase fusion proteins created by genetic engineering retain the binding and catalytic properties of heparinase and of the protein to which it is fused. Three heparinases have been purified to homogeneity from *Flavobacterium heparinum*, and have been produced in a recombinant form in *Escherichia coli*. Fusion proteins consisting of heparinase enzyme combined with binding domains from antibodies or adhesion molecules can be produced with a gene fusion in a recombinant host, while retaining the functionalities of binding and the enzymatic activity of the separate proteins. These molecules can also be purified to homogeneity by procedures normally used for purification of the individual parts of the fusion protein (e.g. affinity chromatography, heparinase purification protocols). Unlike the natural heparinase purified from *Flavobacterium heparinum*, the recombinant enzyme may not contain amino-terminal pyroglutamate or carbohydrate moieties. All recombinant heparinase may contain deletions, additional and/or altered amino acids, which modify the enzymatic activity of the natural enzyme or the functioning of the binding domains. Heparinase and fusion heparinase can be stabilized for *in vivo* use, by complexing with polyethylene glycol, cross linking agents, and by microencapsulation.

For example, the gene for heparinase I was isolated from *F. heparinum* as described by Sasisekharan, et al., *Proc. Natl. Acad. Sci.* 90:3660- 3664, 1993, and an *EcoR* I restriction site was inserted 5' to the codon encoding the glutamine-21

residue by polymerase chain reaction. A fragment containing the heparinase I gene was prepared by digestion with restriction endonucleases; *EcoR* I and *Bam*H I, and ligated to the *EcoR* I/*Bam*H I cleaved pMALc2 plasmid (New England Biolabs). The resulting plasmid contained a hybrid gene encoding a 82,000 - 85,000 Dalton protein incorporating the maltose binding protein (MalB) fused 5' to the heparinase I gene. This plasmid was inserted into *Escherichia coli* HB101 cells using the calcium chloride mediated method described by Cohen et al., *Proc. Natl. Acad. Sci.* 69:2110-211. These cells exhibited heparinase activity under the control of the tac promoter, allowing synthesis of the fusion protein by addition of 0.1 mM of the inducing agent IPTG to the growth medium.

The HB101(pMALc2-HEP1Q21) cells were grown to a cell density of 1.0 g/L dry cell weight in 500 ml, M9 medium containing 0.1 mM IPTG at 37°C and concentrated by centrifugation, 10,000 g x 10 minutes. The cell pellet was suspended in 10 ml 0.025 M Tris, pH 7.7, and the cells disrupted by sonication using a Heat Systems Model XL2020, 4.5 minutes, power level 3, 30 second on 30 second off cycles. Cell debris was removed by centrifugation, 10,000 g x 10 minutes, and the supernatant applied to an amylose affinity resin column (1.0 i.d. x 2 cm, New England Biolabs). The bound protein was eluted with a step gradient of 0.025 M Tris containing 0.01 M maltose at pH 7.5. The fusion protein eluted in a protein peak which displayed a heparinase specific activity of 23.77 IU/mg.

The heparinase-maltose binding fusion protein also can be purified by standard protein separation techniques based on heparinase properties. Cell sonicates were fractionated by ammonium sulfate precipitation. Non-specific proteins were removed with a precipitation step at 1.7 M ammonium sulfate and the supernatant precipitated by raising the ammonium sulfate concentration to 3.2 M. The precipitated material contained the fusion protein and was resuspended in 0.025 M sodium phosphate, pH 6.5. The material was applied to a weak cation exchange column (1.6 i.d. x 10 cm, CBX, J.T. Baker) and eluted with sequential step gradients of 0.0 M sodium chloride, 0.01 M sodium chloride, 0.25 M sodium chloride and 1.0 M sodium chloride, all in 0.025 M sodium phosphate. The fusion protein eluted in the 0.25 M sodium chloride elution fraction and displayed a

heparinase specific activity of 29.95 IU/ml. These two purification procedures demonstrate that functional heparinase fusion proteins can be made by genetically  
5 linking a protein with desired binding properties to the N-terminal end of heparinase and the resulting fusion protein retains the functionality of both heparinase and the protein to which it is fused.

As another example of a fusion protein, a *Bam*H I/*Sal* I restriction fragment  
10 from pGBH3, which contains the heparinase III gene from *Flavobacterium heparinum* was inserted into pMALc2 to form a gene for fusion of a maltose binding protein with heparinase III. Extracts of the *E. coli* strain DH5a containing the fusion gene plasmid were produced as described in the last example, and these extracts contained 18.7 IU/ml/O.D. of heparinase III activity. The extract  
15 was also combined with amylose affinity resin and the resin was then separated from the extract by centrifugation. The resin was washed once with 0.025M Tris (pH 7.5) solution and proteins bound to the resin were resuspended in SDS-PAGE sample buffer and separated according to size on a 7.5% SDS-polyacrylamide gel. Western blot analysis of the gel with anti-heparinase III specific antibody  
20 identified a 116,000 Da. protein, which corresponds to the expected size of the fusion protein. This analysis indicates that the fusion protein has a functional maltose binding domain. This example demonstrates that the heparinase III protein can also be fused to a binding domain to produce a bifunctional fusion enzyme.

#### 25 Protection of Proteins *In Vivo*

Methods for extending the *in vivo* half-life are known and routinely used, especially in the case of enzymes. One example of a suitable method is the attachment of polyethylene glycol moieties to the protein, which inhibits uptake  
30 by the reticuloendothelial system. Preparation and characterization of such non-immunogenic proteins is described by Lu, et al. (*Pept. Res.* 6(3), 140-146, 1993), Delgado, et al. (*Critical Rev. Ther. Drug Carrier Syst.* 9(3-4), 249-304, 1992) and Davis et al. (U.S. Patent Number 4,179,337, 1979), the teachings of which are incorporated herein. Another example of a suitable method is the use of  
35 bifunctional cross-linking agents to stabilize the enzyme against proteolytic degradation. Glutaraldehyde is one type of bifunctional cross-linking agent. PCT



WO95/00171, by Novo Nordisk A/S contains a listing of other useful bifunctional cross-linking agents, and teaches the use of these, which is incorporated herein.

### Preparation of Pharmaceutical Compositions

Heparinase enzyme can be administered either locally or systemically. Local administration can provide greater control. Heparinase is mixed with an appropriate pharmaceutical or veterinary carrier, then administered in an effective amount to produce the desired effect on the treated cells using methods known to those skilled in the art, for example, for local application, use of perfusion, injection or a catheter.

Targeting and effective concentration dosages can be achieved by preparation of targeted enzymes as described above, or by the use of targeting vehicles, such as a catheter or localized injection, to achieve controlled site specific delivery of enzyme.

### Administration of Enzymes via Controlled Release Matrices or Injection

Heparinase enzyme can be formulated in a carrier for administration by injection, for example, in saline or an aqueous buffer, using standard methodology, or encapsulated in a polymeric matrix. Encapsulation of heparinase in controlled release formulations is well known; materials include but not limited to liposomes, lipospheres, biodegradable polymeric matrices, and vesicles. These encapsulants are typically microparticles having a diameter from 60 nm to 100 microns, but preferably less than ten microns, and more preferably one micron or less in diameter.

Proteosomes are prepared from outer membrane proteins of the *Meningococcal* bacteria and been reported to bind proteins containing hydrophobic anchors by Lowell, et al., *Science*, 240:800 (1988). Proteosome proteins are highly hydrophobic, reflecting their role as transmembrane proteins and porins. When isolated, their hydrophobic protein-protein interactions cause them to form naturally multimolecular, membranous 60 to 1000 nm vesicles or membrane vesicle fragments, depending on the strength of the detergent used in their isolation. Heparinase can also be encapsulated within a proteoliposome as described by Miller et al., *J. Exp. Med.* 176:1739-1744 (1992) and incorporated by reference herein, as described above with reference to proteosomes. Alternatively,

heparinase can be encapsulated in lipid vesicles such as Novasome™ lipid vesicles (Micro Vesicular Systems, Inc., Nashua, NH). Another carrier is described  
5 in PCT US90/06590 by Nova Pharmaceuticals, the teachings of which are incorporated herein, which is referred to as a liposphere, having a solid core and an outer shell layer formed of phospholipid.

The carrier may also be a polymeric delayed release system. Biodegradable synthetic polymers are particularly useful to effect the controlled release of  
10 heparinase. Microencapsulation has been applied to the injection of microencapsulated pharmaceuticals to give a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the  
15 cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters and polyamides, particularly those that are biodegradable.

A frequent choice of a carrier for pharmaceuticals is poly  
20 (d,l-lactide-co-glycolide) (PLGA). This is a biodegradable polyester that has a long history of medical use in erodible sutures, bone plates and other temporary prostheses, where it has not exhibited any toxicity. A wide variety of pharmaceuticals including peptides and antigens have been formulated into PLGA  
25 microcapsules. The PLGA microencapsulation process uses a phase separation of a water-in-oil emulsion. Heparinase is prepared as an aqueous solution and the PLGA is dissolved in a suitable organic solvents such as methylene chloride and ethyl acetate. These two immiscible solutions are co-emulsified by high-speed stirring. A non-solvent for the polymer is then added, causing precipitation of the  
30 polymer around the aqueous droplets to form embryonic microcapsules. The microcapsules are collected, and stabilized with one of an assortment of agents (polyvinyl alcohol (PVA), gelatin, alginates, polyvinylpyrrolidone (PVP), methyl cellulose) and the solvent removed by either drying *in vacuo* or solvent extraction. Other means for encapsulation include spray drying, co-precipitation, and solvent  
35 extraction.

### Means for Administration

Heparinase enzyme can be administered by injection, infusion or perfusion.

5 Typically, injection is performed using either a syringe or catheter. Either a syringe or a catheter can be used to apply heparinase locally to areas of blood vessels, tissues or organs. Patients diagnosed with localized inflammations can be treated by introduction of heparinase into their vascular system by these means. Heparinase can also be administered before or simultaneously with  
10 surgery, to reduce resulting inflammatory responses. In addition, preceding transplant surgery, the donor organ can be perfused with a heparinase preparation to reduce inflammation upon reperfusion after transplantation.

The present invention will be further understood by reference to the  
15 following non-limiting examples.

### **Examples**

#### **Example 1: Treatment of Endothelial Cells with Heparinase to Release Heparin/Heparan Sulfate.**

Heparinase alters the cell surface and basement membrane by cleaving the heparin and heparan sulfate moieties from the cell surface and extracellular matrix  
20 proteoglycans. Removal of these glycosaminoglycans will decrease leukocyte-endothelium interactions (leukocyte rolling) by decreasing binding of L-selectin on leukocytes to endothelium proteoglycans. In addition, the removal of heparin and heparan sulfate will decrease binding of chemokines to the endothelium,  
25 which will reduce leukocyte activation, sticking and extravasation. Production of bovine corneal endothelial cells with <sup>35</sup>S- heparin/heparan sulfate proteoglycans and subsequent digestion of these radiolabeled proteoglycans with *Flavobacterium* heparinase III provides a qualitative assessment of the effect of the enzyme on the cell surface. Digestion of cell surface proteoglycans with heparinase III will  
30 release both heparin and heparan sulfate moieties, because these moieties are interspersed on the same unbranched carbohydrate chains. Significant quantities of <sup>35</sup>S- heparin/heparan sulfate were solubilized by the heparinase III treatment.

<sup>35</sup>S-sulfate containing endothelial cell layers were produced by seeding 24 well dishes with primary bovine corneal endothelial cells. These were grown  
35 until 1 day prior to confluence in DMEM with 10% fetal calf serum and 5% calf

serum. One day prior to confluence the cells were diluted 10- fold into Fisher medium supplemented with 10% fetal calf serum, 5% calf serum, 4% dextran, and 25 mCi/ml  $\text{Na}_2^{35}\text{SO}_4$  and cultured for 3 days with the addition of 0.5 ng/ml per day bFGF. Incorporation of label by near-confluent cells localizes the label in and on the cell and minimizes the  $^{35}\text{S}$ - label incorporated into the basement membrane.

Endothelial cell layers containing  $^{35}\text{S}$ - sulfate were treated with 600  $\mu\text{l}$  phosphate buffered saline or heparinases III, in phosphate buffered saline, at a concentration of 1.0 IU/ml, in duplicate wells. The digestions were allowed to proceed for 5, 30 or 60 minutes at  $37^\circ\text{C}$ . After the indicated time of digestion, 400  $\mu\text{l}$  of digestion solution was removed from each well and fractionated on a Bio-sil SEC 125-5 gel filtration column controlled by a Beckman System Gold HPLC, equipped with an autosampler. The flow rate was 1 ml/minute and 1 ml fractions were collected. The amount of  $^{35}\text{S}$ - sulfate present in each fraction was determined by measuring an aliquot of each fraction on a Packard 1600 TR liquid scintillation counter. The labeled, untreated control solutions were fractionated and measured in the same manner, and the quantity of radioactive material in each fraction (background) was subtracted from the amount present in fractions from the heparinase digested samples. The amount of cell surface  $^{35}\text{S}$ -labeled heparin/heparan sulfate in each fraction released by heparinase III treatment is shown in figure 1.

Example 2: Determination of the Extent of Removal and the Rate of Replacement of Heparin/Heparan Sulfate Moieties on Endothelial Cells and in Basement Membranes Treated with Heparinase.

The growth factor, basic Fibroblast Growth Factor (bFGF), is a well characterized heparin binding protein, which is known to bind to the heparin moieties of proteoglycans on the cell surface and in the extracellular matrix (Maccarana, et al., *J. Biol. Chem.*, 268:23898-23905, 1993). Binding of  $^{125}\text{I}$ - labeled bFGF to cell surface and basement membrane proteoglycans was used to access the amount of heparin/heparan sulfate removed from unactivated and IL-1b activated endothelial cell layers and their basement membranes by heparinases I, II or III. Digestion of the cell surface and basement membrane with heparinase will remove both heparin and heparan sulfate moieties, because these moieties are

interspersed on the same unbranched carbohydrate chains. <sup>125</sup>I- labeled bFGF binding was also used in this experiment to determine the rate at which  
5 heparin/heparan sulfate moieties were replaced on the cell surface and basement membrane of endothelium treated with heparinases I, II or III.

Most of the heparin/heparan sulfate can be removed using heparinase (70 to 90%; figures 2, 3 and 4). This demonstrates that the endothelium can be almost  
10 completely depleted of heparin/heparan sulfate moieties by use of heparinase. With a 1 hour heparinase treatment the rate of replacement of the heparin/heparan sulfate is generally biphasic in nature. Replacement of 40 to 50% of the digested heparin/heparan sulfate occurs within a few hours. Additional replacement of the depleted heparin/heparan sulfate occurs at a slower rate  
15 during the remainder of the experiment, with complete replacement occurring 12 to 16 hours. 3 and 5 hour treatments with heparinases caused slower rates of replacement of heparin/heparan sulfate on the cell surface (figures 3 and 4). This was most evident for unactivated endothelium. For the experimental results depicted in figures 3 and 4 for the IL-1 activated endothelium, all three treatment  
20 times (1, 3, and 5 hours) gave lower replacement rates than was observed in the experimental results depicted in figure 2.

This data indicates that significant inhibition of L-selectin binding and immobilized chemokine gradient formation will result from heparinase treatment, and this would result in significant inhibition of localized inflammatory responses.  
25 Also, a treatment period of 3 to 5 hours can greatly extend the period of diminished inflammatory response by decreasing the rate of heparin/heparan sulfate replacement.

A human endothelial cell line was grown to confluency in 48 well dishes in 0.25 ml/well of RPMI medium, containing; 1% penicillin /streptomycin, and  
30 20% fetal serum. Cells in half of the wells were activated for 4 hours with 10 ul of 50 ng/ml IL-1b. After activation all wells were washed 3 times with HBSS and treated with either Incubation Medium (RPMI medium, 25 mM Tris HCL pH 8, 25 mM HEPES pH 7.4, and 0.1% BSA) or 0.1 IU/ml of heparinase I, II or III diluted in Incubation Medium, at 37°C, in 5% CO<sub>2</sub>, for 60 min for experimental  
35 results depicted in figure 2, or for the results depicted in figures 3 and 4, 1.0

IU/ml heparinase I or III diluted in Incubation Medium, at 37°C, in 5% CO<sub>2</sub>, for 60 min with none, 3 or 5 replacements of the enzymes every hour. After enzyme  
5 treatments, the wells were washed 3 times with HBSS and incubated with RPMI medium, containing; 1% penicillin /streptomycin and 20% fetal serum, ± IL-1b, at 37°C, in 5% CO<sub>2</sub>, for the times indicated in Figure 2. The wells were again washed 3 times with HBSS and 0.1 ml of Incubation Medium was added to each well, and the plates were cooled on ice for 5 min. To all of the wells was added  
10 20 ul of 125 ng/ml <sup>125</sup>I-bFGF, and 20 ul of 20 ug/ml unlabeled bFGF. To some of the control wells, 15 ul of 10 mg/ml heparin was also added, to determine nonspecific background binding. The plates were incubated on ice for 40 minutes and washed 2 times with cold HBSS. 0.25 ml of LAB (25 mM HEPES pH 7.4 and  
15 2 M NaCl) was added to each well to solubilize the bFGF, and then it was collected in tubes. This step was repeated, and the contents of the tubes were counted in a gamma counter. The amount of nonspecific background binding was subtracted from each untreated control and treated sample. The sample counts were divided by the control counts to determine the percent of binding that  
20 occurred. The results ± standard errors (SE) are shown in figures 2, 3 and 4.

Example 3: Treatment of Activated Endothelial Cell Layers  
and Basement Membranes with Heparinase to Release  
Heparin/Heparan Sulfate Bound Chemokine, IL-8.

Removal of the bound chemokine gradient formed by activated  
25 endothelium adjacent to inflamed tissue will inhibit the accumulation of neutrophils within this tissue, and will decrease the inflammatory response. The chemokine, IL-8, is produced by endothelium activated by IL-1b and other cytokines and chemoattractants, which are secreted by inflamed tissues. If IL-8 bound to endothelium can be solubilized by treatment with heparinase, then it  
30 would be removed from the area of inflammation by blood flow and the localized inflammatory response would be inhibited. The *in vitro* removal and solubilization of 0.5 to 3 fold more endogenous, immobilized IL-8 (vs. secreted IL-8) from activated endothelium by heparinases I, II or III, or by heparinases I and III demonstrates that the bound chemokine gradient can be destroyed by  
35 heparinase treatment.

One ml of a 3 mg/ml solution of human collagen was used to coat the wells of a 12 well plate. Any remaining collagen solution was removed by aspiration. Human umbilical venous endothelial cells (HUVEC; used at passages 1 to 8) from a confluent 10 ml plate were trypsin treated, and diluted 1 to 7 in RPMI medium containing; 20% fetal serum, 1% penicillin /streptomycin, 100 ug/ml heparin, 10 ug/ml of epidermal growth factor and 200 ug/ml of endothelial cell growth supplement. One ml of diluted cells was added to each well of the collagen coated 12 well plates. The cells were grown until confluent, at 37°C, in 5% CO<sub>2</sub>. The culture medium was changed every other day during the growth period. The day before the chemokine assay the medium was exchanged for 1 ml of RPMI medium without heparin.

To activate the endothelium layer, 50 ng/ml of human recombinant IL-1b, diluted in RPMI medium (minus fetal serum, epidermal growth factor, endothelial cell growth supplement and heparin) and 2% BSA was added to non-control wells to a final concentration of 2 ng/ml. The multiwell plates were incubated at 37°C, in 5% CO<sub>2</sub>, for 4 hours. The medium was removed from all of the wells and the wells were washed two times with Hanks Balanced Salt Solution (HBSS). 0.5 ml of RPMI medium (minus fetal serum, epidermal growth factor, endothelial cell growth supplement and heparin) and 2% BSA was added to the wells for the times indicated in Figure 3. After the indicated times, the wells were emptied and washed once with 1 ml of HBSS. 0.5 ml of HBSS with or without 1 IU/ml of heparinase I, II or III; or heparinases I and III were added to the wells. The plates were incubated at 37°C on a heat block for 15 minutes with occasional agitation. After 15 minutes, the supernatants were collected and assayed for IL-8. An enzyme-linked immunosorbent assay (ELISA) system from Perseptive Diagnostics was used to determine IL-8 concentrations in the supernatants. The manufacturer's recommended protocol was followed. 90 ul of supernatant and 10 ul of 5M sodium chloride were used in each well of the ELISA plate. Each washing step utilized three repeats of 150 ul of washing solution per well, with 2-3 minutes of agitation between each repeat. The percent difference in the IL-8 concentration of supernatants from; IL-1b induced heparinase I, II or III treated endothelium, versus IL-1b induced non-treated endothelium are shown in

Figure 5. In addition, the IL-8 concentrations in the supernatants from IL-1b induced heparinase I, II or III treated endothelium are shown in figure 5.

5 Example 4: Treatment of Endothelial Cell Layers with Heparinase to Inhibit Neutrophil Adhesion.

In order to concentrate neutrophils at a site of inflammation, endothelial cell surface molecules activate rolling neutrophils for tight binding to the walls of postcapillary venules. Chemokines bound to heparin/heparan sulfate have been  
10 identified as important signal molecules for activation of neutrophils for tight binding in the microcapillaries. The *in vitro* neutrophil adhesion assay system described below is commonly used to analyze conditions affecting neutrophil adhesion to endothelial cells. Treatment of the activated human endothelial cell  
15 layers used in this assay with either heparinase I, II or III resulted in significant reductions in the level of neutrophil adhesion to the endothelium. These results demonstrate that heparinase treatment of the vasculature would inhibit localized neutrophil accumulation in the microcapillaries, and would inhibit inflammatory responses.

20 Isolation of Human Neutrophils

25 25 ml of venous blood was drawn from a healthy donor into 1/10 volume of 0.1M sodium citrate, pH 7.4, and was diluted with 25 ml of Dulbecco's phosphate-buffered saline containing calcium chloride and magnesium chloride (D-PBS). 10 ml aliquots of diluted blood were layered on 10 ml of Ficoll-Paque  
30 in 50 ml tubes. The tubes were centrifuged at 400 x g for 30 minutes at 20°C, and were allowed to stop, without braking. The upper layers were removed and the pellets were resuspended in 3 volumes of a solution of 155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub> and 0.1 mM EDTA, pH 7.4 at 4°C, to lyse the erythrocytes. The tubes were inverted after a few minutes and the contents turn black after 7 to 8 minutes.  
35 After 10 minutes, the tubes were centrifuged again at 400 x g for 5 minutes at 4°C. The supernatants were aspirated and the pellets were resuspended in the NH<sub>4</sub>Cl solution containing 0.5% human albumin. The suspensions were pooled and the volume was brought to 50 ml. The cell suspension was incubated on ice for 15 minutes and centrifuged at 400 x g for 5 minutes at 4°C. The supernatant was removed and if the pellet was still red, it was washed again with NH<sub>4</sub>Cl solution



containing 0.5% human albumin. Finally, the cells were resuspended in D-PBS with 5 mg/ml human albumin and refrigerated, until needed. A 10  $\mu$ l aliquot of suspension was diluted with 10  $\mu$ l of trypan blue and the cells were counted on a hemacytometer to determine the number of viable cells per volume of suspension.

#### Labeling of Neutrophils

A suspension of  $10 \times 10^6$  neutrophils was made in 2 ml of PBS (no Ca, no Mg) with 5 mg/ml human albumin. BCECF-AM (Molecular Probes) was added to the suspension for a final concentration of 46  $\mu$ M. The neutrophils were incubated in a water bath at a set temperature of 37°C for 30 minutes after which they were centrifuged and rinsed twice with PBS (no Ca, no Mg) with 5 mg/ml human albumin. They were finally resuspended in 10 ml of RPMI + 20 % bovine fetal serum at 37°C.

#### Treatment of Endothelial Cells with Heparinases

HUVEC at passage number 3 were trypsinized and counted. The cells were plated in RPMI + 20 % bovine fetal serum + 95  $\mu$ g/ml heparin + ECGS + EGF at  $5 \times 10^4$  cells per well in 96 well plates. They were grown at 37 °C, 5% CO<sub>2</sub> for 18 hours. At that point the growth medium was replaced with RPMI + 20 % bovine fetal serum ( 2 ng/ml IL-1b for 4 hours. The cells were then rinsed with HBSS and treated with heparinase I, II or III at 0.1 IU/ml in HBSS for 1 hour at 37 °C, 5% CO<sub>2</sub>.

#### Neutrophil Adhesion Assay

After the treatment period, HUVEC were rinsed once with HBSS. 200  $\mu$ l of the neutrophil suspension (which corresponds to 200,000 neutrophils) was added to each well of treated or control HUVEC. The plate was put at 37 °C, 5% CO<sub>2</sub> for 30 minutes. The adhesion period was stopped by centrifuging the plate upside down at 250 x g for 5 minutes. 200  $\mu$ l of PBS (no Ca, no Mg) + 5 mg/ml human albumin was added to each well and the plate was read with a Fluorolite 1000 fluorescence plate reader at a voltage of 2.5 V. The emission filter was at 535 nm  $\pm$  35 and the excitation filter at 485  $\pm$  22.

#### Analysis of Data

A standard curve was generated by putting known amounts of BCECF-AM-stained neutrophils, resuspended in PBS (no Ca, no Mg) + 5 mg/ml

human albumin in wells of another 96 well plate which contained confluent HUVEC layers. The fluorescence units were plotted against the quantity of neutrophils and a slope was calculated. The standard curve was used to determine the number of bound neutrophils in the control and heparinase treated wells. The percent differences between IL-1b activated HUVEC layers and comparable HUVEC layers treated with heparinases I, II and III are shown in figure 6.

Example 5: Treatment of Endothelial Cell Layers and Basement Membranes with Heparinase to Inhibit Neutrophil Extravasation.

Leukocytes from the blood accumulate in inflamed tissues by transmigration across the adjacent endothelium (extravasation). The endothelial cell layer is activated by the inflamed tissue (via cytokines and chemoattractants), and the affected endothelium directs and localizes the accumulation of leukocytes in the inflamed tissue. In order to activate leukocytes for extravasation, and to direct the migration of the leukocytes into the inflamed tissue, the activated endothelium forms an immobilized chemokine gradient on its cell surface and in its basement membrane. The *in vitro* neutrophil transmigration assay system described below is commonly used to analyze conditions affecting neutrophil extravasation. Treatment of the activated human endothelial cell layers used in this system with either heparinase I, II or III resulted in significant reductions in the level of neutrophil migration across the endothelium. These results demonstrate that heparinase treatment of the vasculature would inhibit localized neutrophil accumulation and inflammatory responses.

Assay of Neutrophil Extravasation

Neutrophils were isolated as described in example 4. Human fibronectin was dissolved at 0.4 mg/ml in RPMI medium without serum. Filter inserts (6.25 mm) of pore size, 3  $\mu$ m or 8  $\mu$ m, were coated with 4  $\mu$ g/cm<sup>2</sup> of human fibronectin for one hour, at room temperature, and were rinsed with distilled water. Wells of a 24 well plate were filled with 0.3 ml of RPMI medium with 20 % fetal bovine serum, 95  $\mu$ g/ml heparin, 200  $\mu$ g/ml ECGS and 10 ng/ml EGF. The coated filter inserts were seated in the wells, and 8 x 10<sup>4</sup> human umbilical venous endothelial cells (HUVEC; used at passages 1 to 7) in 0.3 ml of complete RPMI medium, were

plated on the coated filter inserts. The cells were allowed to grow for 48 to 65 hours, at 37°C, in 5% CO<sub>2</sub>. The culture medium from the filter inserts and the wells was changed once during the growth period for RPMI medium lacking heparin. After the growth period, the culture medium underneath all inserts, except negative control inserts, was removed and replaced with fresh culture medium lacking heparin and growth factors, but containing 2 ng/ml of human recombinant IL-1b. The culture medium under negative control inserts was replaced with fresh culture medium. The multiwell plates were incubated at 37°C, in 5% CO<sub>2</sub>, for 4 hours. The medium was removed from the inserts and wells and the cells were rinsed once with Hank's Balanced Salt Solution (HBSS). The filters and wells were filled with 0.3 ml of a solution of HBSS; treated inserts received HBSS containing heparinase I, II or III instead of HBSS. This treatment was performed for the times indicated in Figure 1, at 37°C, in 5% CO<sub>2</sub>. The solution was removed and the cells were rinsed once with HBSS. 0.3 ml of fresh culture medium without heparin and growth factors was added to the wells and 1.5 x 10<sup>6</sup> of freshly prepared human neutrophils in 0.3 ml of culture medium without heparin and growth factors were added to all of the inserts. The plates were incubated at 37°C, in 5% CO<sub>2</sub>, for the times indicated in Figure 3. After this time, the inserts were removed and the bottoms were rinsed once with 0.3 ml D-PBS. The contents of the well were removed and the well was washed with 0.3 ml of D-PBS. The rinses were added to the well contents and the combined contents were brought to a 1 ml volume. These samples were frozen for up to 16 hours before assaying for myeloperoxidase activity.

#### Myeloperoxidase Assay

Standards containing between 1 x 10<sup>5</sup> and 1 x 10<sup>6</sup> neutrophils in a 1 ml volume were produced by diluting an aliquot of the neutrophil suspension in D-PBS. 4 ml of 50 mM potassium phosphate, pH 6.7, containing 0.5 % hexadecyltrimethylammonium bromide and 0.5 % triton was added to each standard and thawed sample. 0.1 ml of each sample or standard was added to a plastic cuvette. 2.9 ml of 50 mM phosphate buffer, pH 6.0 containing 0.167 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide were added to the

cuvettes. The change in absorbance at 460 nm was monitored every 30 seconds for 3 minutes by use of a spectrophotometer.

5 The rate changes obtained from the standards were used to produce a curve of the rate of increase in absorbance versus numbers of neutrophils. This curve was used to quantitate the number of neutrophils in each sample, which had migrated through, either a treated, or an untreated endothelial layer. The number of migrating neutrophils was divided by  $1.5 \times 10^6$  to determine the  
10 percentage of neutrophil migration.

#### Analysis of Data

The effectiveness of the heparinase treatment in this assay depended on the extent to which the HUVEC were covering the filter surface. The extent of  
15 coverage was based on dye exclusion analysis performed after an extravasation experiment, and it varied somewhat from filter to filter in a single experiment. Generally, if the filter was densely covered with a tightly packed HUVEC layer, the percentage of extravasating neutrophils was low ( $<10\%$ ), and the differences between treated and untreated wells were not statistically significant (large well  
20 to well variability). If large areas of the filter were not covered with a HUVEC layer (estimated at 30-40% of the filter) large numbers of neutrophils (40-60%) would migrate through the filter, but a 1 hour heparinase treatment would not be as effective in inhibiting the migration. This migration is not comparable to extravasation, which can be functionally defined as neutrophils squeezing between  
25 neighboring endothelial cells. If 75 to 90% of the filter was covered with HUVEC, generally, 15 to 30% of the neutrophils extravasated, and the 1 hour heparinase treatment was found to be most effective under these conditions.

Using the Student's t-test, experiments were analyzed to determine if a significant effect for the 1 hr heparinase treatments had been observed ( $P < 0.05$ ).  
30 The data from these experiments was combined and is displayed in figure 7. The data for the 15 minute heparinase treatments shown in figure 7 is derived from the same experiments as the 1 hour treatments. The Student-Newman-Keuls test was used to determine the significance of the differences ( $P < 0.05$ ) between  
35 different treatments with the same enzyme. Significant differences are indicated by asterisks (\*) in figure 7.

Example 6: Treatment of Endothelial Cell Layers and Basement  
Membranes with Human Heparinase (b-thromboglobulin)  
to Inhibit Neutrophil Extravasation.

5 Commercial preparations of b-thromboglobulin are a mixture of the chemokines CTAP-III and NAP-2. At non-physiological pH (pH 5.8-6), these chemokines have heparinase activity, while at pH 7, they bind heparin and act as chemotactic cytokines for leukocytes. The heparinase activity of commercial  
10 b-thromboglobulin preparations were analyzed at pH 5.8 and pH 7, by digestion of radioactive  $^{35}\text{SO}_4$ -labeled ECM. These preparations showed significant human heparinase activity only at pH 5.8. They were then used in the *in vitro* extravasation assay system described in example 5, in order to determine if the human heparinase activity could prevent extravasation of neutrophils across an  
15 endothelial cell layer. The treatment of activated human endothelial cell layers with human heparinase (b-thromboglobulin) resulted in a significant reduction in neutrophil extravasation. These results demonstrate that heparinase treatment of the vasculature with human heparinase would inhibit localized neutrophil accumulation and inflammatory responses.

20 Activity of b-thromboglobulin on labeled matrix

Commercial b-thromboglobulin was tested for heparinase activity at pH 5.8 and pH 7 by release of  $^{35}\text{SO}_4$ -labeled heparin/heparan sulfate from ECM. Bovine corneal endothelial cells at passage 1 to 8 were split 1:10 from a confluent plate and seeded in 4 well plates in DMEM low glucose with 10 % fetal bovine serum,  
25 5% calf serum and 4% Dextran added. The dishes received 1 ng/ml bFGF 3 times a week prior to reaching confluency. Just prior to reaching confluency,  $\text{Na}_2^{35}\text{SO}_4$  in  $\text{H}_2\text{O}$  diluted to 1 mCi/ml with DMEM low glucose was added to the cells at a final concentration of 25 uCi/ml, in Fisher medium with 10 % fetal bovine  
30 serum, 5 % calf serum and 4% Dextran. 3 to 4 days later, the label was given again. The plates were left undisturbed 12 to 14 days post confluency. To harvest the matrix, the medium was removed and replaced with a solution of 0.5% Triton, 0.02M  $\text{NH}_4\text{OH}$  in PBS (no Ca, no Mg). This solution was removed and the matrix was washed 3 times with PBS (no Ca, no Mg). The plates were stored at 4°C,  
35 covered with PBS. These matrices were used within a year.

b-thromboglobulin from Wellmark (product #41705) or Calbiochem (product #605165) were used for digestion of the labeled ECM. 100 ug of enzyme  
5 was dissolved in 1 ml of water (Wellmark) or PBS (Calbiochem). A further  
dilution was done in PBS at pH 5.8 or 7. The matrices were covered with 250 ul  
of PBS alone or PBS with 1 or 5 ug of  $\beta$ -thromboglobulin. The matrices were  
incubated in a CO<sub>2</sub> incubator for 3 hours. Aliquots of 100 ul were taken from  
every well and counted. The amount of radioactivity released from each  
10 enzyme-treated matrix was compared to an untreated matrix, and the results are  
displayed in figure 8.

#### Migration of neutrophils across HUVEC treated with $\beta$ -thromboglobulin

HUVEC were grown on filter inserts and activated as described in example  
15 5. Five ug of  $\beta$ -thromboglobulin in PBS at pH 5.8 or PBS alone were applied to  
the HUVEC layer for one hour. Neutrophils were added above the filters and the  
number of extravasating neutrophils were quantitated as described in example 5.  
The effect of the human heparinase treatments on neutrophil extravasation are  
shown in figure 9.

#### 20 Example 7: Treatment of Rats with Heparinase Inhibits Leukocyte-Endothelial Cell Interactions Following Ischemia/Reperfusion.

This example illustrates the effect of heparinase III on leukocyte behavior  
*in vivo*. Three key mechanisms of leukocyte accumulation in inflamed tissues;  
leukocyte rolling, sticking, and extravasation, were analyzed in rat skeletal muscle  
25 microvasculature following ischemia. Pretreating the vasculature with heparinase  
III prior to ischemia, and maintaining the plasma concentration of heparinase III  
constant during reperfusion was found to significantly decrease leukocyte rolling,  
sticking and extravasation. This example demonstrates that heparinase treatment  
of vasculature would inhibit neutrophil accumulation in microcapillaries and in  
30 the surrounding tissues. *In vivo* heparinase treatment would result in decreased  
inflammatory responses. As also demonstrated by this example, heparinase  
treatment significantly increased microvascular perfusion within reperfused  
muscle following ischemia. In addition to decreased neutrophil accumulation,  
35 increased microvascular perfusion would positively affect the recovery of muscle

tissue and positively affect the outcome of ischemia/reperfusion (i.e. inflammatory) events.

#### General Comments

In order to establish a plasma level of approximately 1 IU/ml, we undertook pilot studies to test the effect of infusing Heparinase over the 5 hour period of time required for our *in vivo* studies. A previous publication on the leukocyte behavior following 3 hr ischemia ( Forbes et al., 1996, Microvascular Research 51:275-287) contains data on naive and sham treated rats. Because the methods and timing were the same for this previous study and the present study of 3 hr ischemia, the effect of heparinase treatment will be compared to the naive and sham results obtained from the previously published results. For the 2 hr ischemia protocol, additional naive and sham treated rats were analyzed.

In order to investigate directly the effect of heparinase, *in vivo*, we applied intravital video microscopy to the extensor digitorum longus muscle in the rat hind limb. This analysis occurred during a period of 105 or 90 minutes of reperfusion, which followed 2 or 3 hours, respectively, of no-flow ischemia. Such periods of ischemia/reperfusion (I-R) result in an inflammatory response in skeletal muscle sufficient to cause increased leukocyte-endothelial cell interactions.

#### Methods

Male Wistar rats weighing 225 to 250 gm were anesthetized by inhalation of halothane (1% - 1.5%) and the carotid artery and jugular vein cannulated to monitor blood pressure and permit infusion of fluids, respectively. The extensor digitorum longus (EDL) muscle in the rat hind limb was prepared for intravital microscopy. Briefly, with the anaesthetized rat lying on the stage of the microscope, the EDL muscle was exposed by reflection of the overlying skin and separation of the tibialis anterior and gastrocnemius muscles. A suture was tied around the distal tendon of the muscle allowing reflection of the muscle into a saline bath positioned on the microscope stage. Normal muscle and body temperatures were maintained (i.e., muscle at 32°C; body at 37°C) by heat lamps. The muscle was covered by a glass cover slip and all exposed tissue covered with Saran wrap to prevent dehydration. Following the preparation of the EDL muscle for intravital video microscopy, and a 30 minute period of recovery, to allow

microvascular blood flow to return to normal following the hyperaemia induced by the preparatory methods, 1-2 fields of view each containing two or more postcapillary venules were chosen. These fields of view were used throughout the experiment so that temporal changes in leukocyte flow behavior could be measured. One minute recordings of these fields of view were made using low magnification to provide information regarding RBC flow within individual capillaries. Following the low magnification recordings, views of the postcapillary venules were recorded for 1 min at high magnification. The video recording of such views allows for the "off-line" analysis of microcirculatory parameters. Thus, control values of the density of perfused capillaries and the flow behavior of leukocytes (number of stuck, rolling and extravasating per unit area) were measured.

Plasma levels of heparinase III are measured using a heparinase III ELISA. The heparinase III ELISA is a quantitative two-antibody sandwich assay. Affinity purified anti-heparinase III rabbit antibodies are coated onto a microtiter plate. Wells are washed and incubated for 2 hours at 37°C with blocking buffer (TBS, 1% BSA +1% Tween 20). After 3 washes, standards and samples are added to the wells and any heparinase III present is bound by the immobilized antibody. Any unbound substance is then washed away and biotin labeled anti-heparinase III rabbit antibodies are added to the wells. Excess antibodies are removed by washing. Peroxidase-conjugated streptavidin is added and binds to any biotin complex present in the well. After washing away any unreacted streptavidin, a substrate solution containing hydrogen peroxide and 3, 3', 5, 5' tetramethylbenzidine in aqueous DMF is added to the wells, according to the procedure described for the TMB Peroxidase EIA Substrate Kit (Biorad, CA), and color develops in proportion of the amount of heparinase III in the sample. A 1 N H<sub>2</sub>SO<sub>4</sub> solution stops the reaction and the absorbance is measure at 450 nm.

#### Specific Protocol

A series of rats were infused with heparinase III via the venous catheter at a rate of 3 ml/hr for 5 hr. to maintain a heparinase level of 1.0 IU/ml in the blood. Through such pilot studies it was determined that 0.33 IU/gm body weight/hr was adequate for this purpose.



Microvascular blood flow and leukocyte behavior was recorded every 15 min for a total of 90 to 105 min of reperfusion. Blood samples were taken before  
5 the administration of heparinase and during reperfusion to ensure correct plasma concentrations of heparinase.

### Statistical Analysis

In all cases means are expressed with their standard error of estimate. Comparisons were made using analysis of variance (ANOVA) followed where  
10 appropriate by Scheffé tests as ad hoc analysis. Significance was assumed at  $p > 0.05$ .

### Results

A plasma concentration of approximately 1 IU/ml ( adjusted for activity)  
15 was achieved during infusion, and in spite of a trend toward increased plasma levels during the last 2 hours of infusion, the plasma concentration remained constant (figure 10). Prolonged infusion of heparinase III appeared to have no adverse side-effects at least in terms of blood pressure, or rate of respiration.

Immediately following 3 hr of ischemia, the number of rolling leukocytes  
20 (Lr) significantly increased in sham I-R rats ( $14.77 \pm 1.33$ ), compared to naive (no I-R) rats ( $5.66 \pm 0.11$ ). The average number of rolling leukocytes remained constant at these levels during reperfusion in both the sham and naive rats for the duration of the 90 minute reperfusion period (Figure 3). In spite of 3 hr ischemia, no change in leukocyte rolling within postcapillary venules of heparinase III  
25 treated rats was measured. In fact, the average number of rolling leukocytes following heparinase treatment ( $2.81 \pm 0.64$ ) tended to be less than in naive rats over the 90 minute observation period (figure 11).

The number of leukocytes stuck to the wall of postcapillary venules (Ls)  
30 progressively increased in sham I-R rats, reaching a constant level ( $11.96 \pm 0.01$ ) within 45 minutes of the release of the tourniquet (3 hr ischemia). However, the number of stuck leukocytes in heparinase III treated rats showed no change following ischemia, and was not significantly different from naive rats (figure 12).

As would be expected based on the low numbers of sticking leukocytes  
35 following heparinase III treatment, the number of extravasated leukocytes (Le) did not change during reperfusion after 3 hr of ischemia (figure 13). Le for sham and

naive rats are not available for the 3 hr ischemia protocol. For a 2 hr ischemia protocol, the Le for the heparinase III treated rats was higher than for the naive rats, but was significantly lower than the Le in the sham treated animals. This data is displayed in figure 14, as the percent difference in the Le for heparinase and sham treated rats vs. naive rats.

Following release of the tourniquet after 3 hr of ischemia, a significant decline in microvascular perfusion (CDper) occurred in both the sham and heparinase III treated rats, compared to perfusion measured in naive rats. However, unlike the perfusion in sham rats microvascular perfusion in heparinase III treated muscles returned to normal within 30 minutes of the release of the tourniquet (figure 15).

Example 8: Cardioprotective effects of Heparinase III in a rabbit preparation of ischemia/reperfusion injury.

### Introduction

Ischemia produces a significant degree of damage at the level of myocytes and endothelial cells within the coronary vascular bed; this can lead to extravasation of plasma and other blood and cellular components into the interstitial space. Polymorphonuclear leukocytes can migrate through the endothelial cell layer; this migration of neutrophils across the connective tissue barrier is dependent on the actions of neutrophil-derived proteolytic enzymes even in the presence of plasma antiproteases. Restoration of blood flow allows rapid access of inflammatory cells to jeopardized myocardium. Neutrophil adhesion to endothelial cells is stimulated by endotoxin IL-1b (activates vascular endothelium to produce adherence molecules for leukocytes) or tumor necrosis factor. A number of studies have explored the possibility of using various pharmacologic interventions including monoclonal antibodies to prevent neutrophil adhesion to vascular or myocardial cells particularly during the reperfusion phase. Interfering with neutrophil-cellular interactions (neutrophil rolling, adhesion and extravasation) has been shown to significantly reduce the extent of cellular injury following ischemia-reperfusion. This suggests that inflammatory cells play an important role in the pathophysiology of ischemia-induced cellular injury; inflammatory cells may also play a role in extending myocyte injury beyond that

which occurs during the ischemic insult (i.e., reperfusion injury). Because heparinase treatment is effective for inhibition of neutrophil-endothelium interactions (see examples above), prevention of reperfusion injury to rabbit myocardium by treatment with heparinase was investigated in the experiments described in this example. Heparinase III was found to attenuate the extent of tissue necrosis when administered at a target dose of 25 ug/ml either before the onset of coronary occlusion or at the onset of reperfusion. Lower dosages of heparinase III did not provide cardioprotection in this animal preparation of ischemia-reperfusion injury; however, at a target dose of 5 ug/ml there was a trend toward reduced infarct size. Protection was obtained without significant changes in cardiac hemodynamics or transmural blood flow distribution.

When an inflammatory response is the result of an ischemic episode as in the non-limiting examples of heart attack and stroke, this example demonstrates that heparinase treatment before or at the time of the inflammatory event can reduce tissue injury. In addition, this example indicates that tissue damage resulting from leukocyte accumulation during any inflammatory event can be reduced by heparinase treatment or pretreatment.

#### Methods

Male New Zealand White rabbits (2.2-3.0 Kg body weight) were used for these studies. Rabbits were cared for in accordance with the *Guide to the Care and Use of Experimental Animals (vol. 1 and 2)* of the Canadian Council on Animal Care. They were premedicated with intramuscular acepromazine maleate (5 mg/Kg; Austin Laboratories) and anesthetized with pentobarbital sodium (25 mg/Kg; i.v.; MTC Pharmaceuticals). Additional anesthetic was administered hourly. The trachea was cannulated and rabbits were mechanically ventilated with room air. The right jugular vein was cannulated for administration of drugs (Heparinase III or vehicle); the left jugular vein was cannulated to permit withdrawal of blood for determinations of plasma heparinase levels. A cannula (PE-90) was placed in the left carotid artery for withdrawal of reference arterial blood during injection of radiolabeled microspheres.

The heart was exposed via a left thoracotomy and a snare (4-0 silk) placed around the first anterolateral branch of the left circumflex coronary artery midway

between the atrioventricular groove and the apex. The silk suture was passed through a length of Tygon tubing to provide a snare for coronary occlusion. Left  
5 ventricular chamber pressure was obtained with a fluid-filled catheter positioned via the apex. Cardiac hemodynamics were allowed to stabilize for 20 minutes.

Regional myocardial ischemia was induced by pulling the suture through the plastic tubing and clamping with a mosquito hemostat. Ischemia was verified  
10 visually by the appearance of regional epicardial cyanosis and ST segment elevation on the electrocardiogram (Lead II). In hearts that developed ventricular fibrillation normal sinus rhythm was restored by gentle flicking of the LV (electrical cardioversion was not used in these experiments); hearts that could not  
15 be cardioverted after two attempts were excluded from the data analysis. Lead II electrocardiogram and LV pressure were recorded throughout the experiments with a Gould (TA240) EasyGraph 4-channel physiograph recorder (Interfax Inc., Montreal, Quebec).

#### Experimental Protocol

Rabbits were assigned to seven different treatment groups; Group 1 rabbits  
20 were given saline (i.v.) for 60 minutes prior to onset of ischemia; Group 2 rabbits were given saline (i.v.) at the onset of coronary reperfusion; Group 3 rabbits were given heparinase III (25 ug/ml target level, i.v.) for 60 minutes prior to onset of ischemia; Group 4 rabbits were given heparinase III (25 ug/ml target level, i.v.) at the onset of coronary reperfusion; Groups 5, 6 and 7 rabbits were given target  
25 levels of either 0.25, 1.25, or 5.0 (g/ml heparinase III, respectively at the onset of coronary reperfusion. Drug or saline was infused intravenously (4.0 ml/hr) for 60 minutes prior to onset of myocardial ischemia in two treatment groups (Groups 1 and 3) and then continuously for 2 hours using a Harvard infusion pump (Ealing Scientific, Montreal, Canada). In the remaining experimental groups  
30 vehicle or drug infusion was initiated at the time of reperfusion and continued for 3 hours during reperfusion. Rabbits were assigned to a particular group by rotating drug treatment on succeeding experiments through the seven treatment protocols. All rabbits were subjected to 45 minutes of regional coronary occlusion  
35 followed by 180 minutes of reperfusion.

### Plasma heparinase III Determinations

Blood samples were obtained from the right jugular vein at baseline, 30 and  
5 60 minutes after vehicle or drug infusion in Group 1 and 3 rabbits; blood was also  
obtained at 15, 30, 60, 120 and 180 minutes of coronary reperfusion. In the  
remaining experimental groups blood was obtained at baseline and 15, 30, 60, 120  
and 180 minutes of coronary reperfusion. Blood samples were centrifuged for 15  
minutes at 1500 rpm at 4°C; plasma was frozen and stored at -20 °C for later  
10 analysis. Heparinase III plasma levels were determined as described in example

### 7. Transmural Blood Flow

Blood flow to ischemic and non-ischemic vascular beds was measured  
using radiolabeled microspheres ( $\pm 15 \mu\text{m}$ ; NEN, Boston, MA) using the reference  
withdrawal technique. For each blood flow measurement,  $0.4\text{--}0.6 \times 10^6$   
15 microspheres labeled with either  $^{113}\text{Sn}$ ,  $^{46}\text{Sc}$ , or  $^{85}\text{Sr}$  (agitated mechanically with  
a vortex mixer immediately before use) were injected into the left atrium under  
hemodynamic steady-state conditions followed by two flushes with 3 ml warmed  
saline (injection of microspheres into the left atrium ensures adequate mixing in  
the LV chamber and prevents streaming artifacts which occur with direct  
20 injections of microspheres into the coronary circulation). Reference arterial blood  
samples from the carotid artery were collected beginning 10 seconds before the  
injection of microspheres and continuing for 2 minutes thereafter at a rate of 2.6  
ml/min. Myocardial blood flow distribution was assessed at; 1- baseline, 2- 30  
25 minutes after onset of coronary reperfusion and 3- 180 minutes coronary  
reperfusion. Tissue and reference blood radioactivity is measured using a  
multichannel pulse-height analyzer (Cobra II, Canberra Packard) with correction  
for overlap of isotope spectra.

### Analysis of Infarct Size

30 At the end of the experimental protocol, hearts were arrested in diastole by  
intravenous injection of 10 ml of saturated potassium chloride, quickly extirpated,  
rinsed in saline and cannulated via the aorta on a Langendorff perfusion  
apparatus. Hearts were perfused *ex vivo* via the aorta at 75 mm Hg with  
2,3,5-triphenyltetrazolium chloride at 37°C for 20 minutes. Subsequently, the  
35 arterial suture was re-tied and Monastral Blue was injected retrogradely via the

aortic cannula to allow delineation of the anatomic risk zone. Hearts were then removed from the perfusion apparatus, the atria and right ventricle were trimmed away, and the left ventricle was weighed and fixed by immersion in buffered 10% formalin.

#### Post-mortem Studies

The principal end-point of this study was the effect of drug treatment on infarct size (normalized to anatomic risk zone size), assessed using tetrazolium staining. Hearts were sectioned into 2 mm slices and the outline of the LV slices and the tetrazolium-negative (i.e., infarct) areas were traced onto clear acetate sheets. Anatomic risk zone was delineated by the absence of Monastral blue dye and traced onto clear acetate sheets. Infarcts were normalized to anatomic risk zone size for each heart. Total LV cross-sectional area, risk area, infarct area were determined from enlarged tracings (1.5X) by computerized planimetry (Sigma Scan; Jandel Scientific Inc., Calif.) using a Summagraphics Summasketch Plus Bitpad connected to an IBM PS/2 computer. Risk volume, infarct volume, and LV volume for each slice was calculated as the sum of the area obtained by computerized planimetry and the thickness of each ventricular slice. The values from the sequential slices were summed to provide the total volume of the LV, risk zone and infarct zone.

#### Data Analysis

Differences in hemodynamic data before and after coronary occlusion were examined using one-way ANOVA. Heart rate-blood pressure product was used as an index for cardiac metabolic demand. Infarct volume, risk volume, infarct size, and LV volume were compared by a one-way ANOVA. Where overall group differences were detected, Dunnett's multiple comparison test was used for comparison to controls. All statistical comparisons were made with Statistical Analysis Systems Programs (SAS Institute, Cary, NC) for the personal computer. A probability ( $p$ ) level of less than 0.05 was considered statistically significant. To establish sample size for this study, "n/group" values were calculated to provide a 0.90 power to detect a minimum 15 percent reduction (expected standard deviation of 8 %) of infarct size.

## Results

One hundred thirty rabbits were entered into the present studies; five rabbits died due to respiratory failure (n=1), surgical error (n=1), or non-convertible ventricular fibrillation (n=3). Thirty-six rabbits were included in the dose-response studies and another 28 were assigned to the biochemical evaluations (cardiac hemodynamic and plasma heparinase III levels were included in the overall statistical analysis). Consequently, sixty-one rabbits were included in the infarct size data analyses.

Cardiac hemodynamic variables are summarized in Table 1. Heart rate, left ventricular systolic and diastolic pressures and heart rate-blood pressure product (indicator of myocardial oxygen demand) before the onset of coronary occlusion were comparable for all of the treatment groups. Heart rate-blood pressure product (figure 16) appeared to be higher at 60 minutes reperfusion in rabbits treated with heparinase III (25 ug/ml target level) at the time of reperfusion (Group 4); however, cardiac hemodynamics were similar for all animals at the end of the study.

Data on left ventricular weight, infarct and risk volume, infarct size and anatomic risk area as percent of LV volume are summarized in Table 3. Infarct size (figure 17), normalized to risk zone size, was  $42.3 \pm 4.8$  (mean  $\pm 1$  SD) and  $40.0 \pm 5.3$  percent (p=NS) in controls with/without vehicle pre-treatment, respectively. Heparinase III pretreatment and heparinase III given at the time of reperfusion at the 25 ug/ml target level resulted in a significant reduction (p=0.01 versus vehicle treated controls) in myocyte necrosis of  $26.1 \pm 5.2$  and  $24.7 \pm 5.1$  percent, respectively; no differences were detectable between groups treated either pre-ischemia or at the time of coronary reperfusion. Treatment with heparinase III at 0.25, 1.25 or 5.0 ug/ml target levels did not limit infarct size; they were  $42.8 \pm 6.5$ ,  $39.1 \pm 5.4$ , and  $37.9 \pm 4.6$  per cent respectively. There was a slight trend to smaller infarcts in the 5.0 (g/ml treatment group with a p value of 0.066.

Heparinase III injectate levels which were administered to the respective treatment groups are shown in figure 18. The initial heparinase III target dose (i.e., therapeutic dose) of 25 ug/ml was investigated followed by the respective drug dilutions of 1:5, 1:20 and 1:100; dilutions were made with saline. A

time-course of actual plasma heparinase III concentrations is shown in figure 19; pre-treatment with heparinase III and treatment at the time of reperfusion  
5 provided similar plasma drug concentrations in Groups 3 and 4. Plasma  
heparinase III concentrations were considerably lower after 3 hours coronary  
reperfusion in rabbits which were initially pre-treated (drug only administered  
during first 3 hours of experimental protocol); most importantly, the drug was on  
board at the time of coronary reperfusion. This may account for the similar  
10 results obtained in Groups 3 and 4 with respect to infarct size.

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**Table 1. Summary of Cardiac Hemodynamics**

	Group	HR (bpm)	LVPsys (mm HG)	LVPdias (mm HG)	RPP (mm Hg x bpm)
5					
		<u>Baseline</u>			
	1	229±34	63±8	2±1	14,192±3,643
	2	245±31	58±10	2±2	13,705±2,739
10	3	246±29	64±10	3±3	15,169±3,629
	4	242±39	70±12	3±2	16,276±4,180
	5	254±27	67±11	2±2	16,432±2,718
	6	231±34	63±12	1±1	14,389±4,116
15	7	233±28	67±12	1±1	15,293±3,757
		<u>Ischemia (30 minutes)</u>			
	1	218±26	57±8	4±3	11,518±2,904
	2	237±33	49±11	4±3	10,833±3,372
	3	241±29	60±8	6±4	11,586±2,634
20	4	236±40	64±11	5±4	14,216±3,832
	5	228±31	61±12	2±2	13,421±3,545
	6	219±34	60±13	4±3	12,639±4,551
	7	211±26	58±8	4±2	11,434±2,704
25		<u>Reperfusion (30 minutes)</u>			
	1	206±41	57±8	3±2	11,257±3,731
	2	229±43	50±9	3±2	11,006±3,755
	3	201±32	60±10	5±3	11,129±3,069
30	4	238±33	64±11	5±3	14,218±4,031
	5	206±24	61±11	3±3	12,166±2,833
	6	197±32	59±11	2±1	11,523±3,825
	7	191±22	57±11	3±2	10,447±2,888
		<u>Reperfusion (60 minutes)</u>			
35	1	199±26	57±7	3±2	10,921±2,695

	Group	HR (bpm)	LVPsys (mm HG)	LVPdias (mm HG)	RPP (mm Hg x bpm)
5	2	220±42	52±10	3±2	11,022±3,932
	3	197±28	61±13	5±3	11,038±2,981
	4	233±34	67±12	5±3	14,313±4,082
	5	199±27	60±11	2±2	11,467±3,172
	6	182±31	59±11	2±2	10,416±3,151
10	7	183±22	59±10	4±3	10,091±2,138
	<u>Reperfusion (120 minutes)</u>				
15	1	177±22	57±8	2±1	9,701±2,499
	2	197±40	52±10	3±2	9,745±3,870
	3	188±29	62±12	5±3	10,736±3,180
	4	210±37	65±13	6±4	14,313±4,082
	5	184±31	61±13	2±2	10,967±3,624
	6	165±26	59±11	2±2	9,366±2,587
20	7	165±21	56±10	3±3	8,926±2,164
	<u>Reperfusion (180 minutes)</u>				
25	1	156±32	53±13	2±1	8,188±3,450
	2	187±46	49±5	4±3	8,482±2,163
	3	178±36	58±10	4±2	9,573±2,591
	4	206±40	65±15	7±4	11,924±4,116
	5	172±35	58±12	2±2	9,899±3,542
	6	154±28	59±12	2±2	9,123±3,313
	7	154±22	55±10	2±2	8,162±2,346
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Values are means ±1 SD. HR = heart rate; LVPsys = systolic left ventricular chamber pressure; LVPdias = diastolic left ventricular chamber pressure; RPP = heart rate-blood pressure product.

**Table 2. Myocardial Blood Flow in Ischemic and Non-ischemic Perfusion Beds**

Group	Ischemic perfusion bed			Non-ischemic perfusion bed		
	BASE	30" RP	180" RP	BASE	30" RP	180" RP
1	2.17±0.99	1.90±0.57	1.02±0.63	1.91±1.11	2.20±0.78	1.22±0.64
2	2.73±1.31	1.64±0.75	0.88±0.32	2.52±1.07	1.92±0.71	1.38±0.23
3	2.13±1.11	1.24±0.711	1.17±0.96	2.38±0.78	1.78±0.51	1.71±0.69
4	2.33±0.83	1.68±0.87	0.84±0.47	2.52±0.71	2.24±0.64	1.42±0.46
5	2.76±1.15	1.98±0.65	1.27±0.55	2.74±1.55	1.83±0.55	1.36±0.28
6	2.43±0.70	2.41±0.38	1.57±0.58	2.19±0.68	2.24±0.36	1.46±0.29
7	2.38±0.45	2.14±0.76	1.13±0.49	2.41±0.58	2.20±0.61	1.39±0.35

Values are means ±1 SD. <sup>1</sup>p(0.05 versus Group 6. BASE = baseline flow measurement (i.e., before ischemia); RP = coronary reperfusion. Data are expressed in ml/min/g wet weight.

**Table 3. Infarct Size Measurements**

Group	n	Hwt (g)	AN (cm <sup>3</sup> )	AR (cm <sup>3</sup> )	ANAR (%)	ARLV (%)
1	9	4.46±0.28	0.51±0.11	1.22±0.27	42.3±4.8	36.5±6.7
2	9	4.53±0.63	0.41±0.07	1.04±0.18	40.0±5.3	33.9±5.8
3	9	4.71±0.69	0.26±0.06 <sup>1</sup>	1.03±0.31	26.2±4.9 <sup>1</sup>	33.3±8.1
4	9	4.51±0.77	0.24±0.07 <sup>1</sup>	0.98±0.24	24.7±5.1 <sup>1</sup>	31.7±7.2
5	8	4.30±0.52	0.36±0.12	0.91±0.12	42.8±6.5	29.4±4.6
6	9	4.61±0.52	0.39±0.08	1.02±0.27	39.1±5.4	32.0±7.9
7	9	4.02±0.63	0.30±0.05	0.80±0.18 <sup>1</sup>	37.9±4.6	27.5±6.7

Values are means ±1 SD. <sup>1</sup>p(0.05 versus controls. Hwt = ventricular weight; AN = area of necrosis, AR = area at risk; ANAR = necrosis normalized to anatomic risk area; ARLV = risk zone normalized to total LV area.

These data indicate the utility of compositions containing heparinase for diminishing localized inflammatory responses.

5        Modifications and variations of the compositions and methods of use of the present invention will be obvious from this detailed description to those skilled in the art. Such modifications are intended to come within the scope of the following claims.

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What Is Claimed Is:

1. A method to decrease localized inflammatory responses in a tissue  
5 of a patient comprising administering to said patient heparinase enzyme in an effective amount sufficient to decrease said localized inflammatory response.
2. The method of claim 1, wherein said administration of said heparinase enzyme removes and digests heparin and heparan sulfate from endothelial cell surfaces and extracellular matrices of said tissue.  
10
3. The method of claim 1, wherein said administration of said heparinase enzyme decreases the accumulation of leukocytes in tissue adjacent to endothelial cell surfaces and extracellular matrices.
4. The method of claim 1, wherein said administration of said  
15 heparinase enzyme inhibits leukocyte extravasation by releasing immobilized chemokines and destroying chemokines immobilized to endothelium.
5. The method of claim 1, wherein said administration of said heparinase enzyme inhibits leukocyte rolling on endothelium.
6. The method of claim 1, wherein said heparinase enzyme is  
20 overexpressed from a recombinant nucleotide sequence, in *Flavobacterium heparinum*.
7. The method of claim 1, wherein said heparinase enzyme is expressed from a recombinant nucleotide sequence in an organism in which it does not naturally occur.
- 25 8. A method to decrease a localized inflammatory response in a tissue of a patient comprising administering to said patient a fusion protein comprising a ligand which binds to activated endothelial cells and a heparinase enzyme in an amount sufficient to decrease said localized inflammatory response in said tissue.
9. The method of claim 8, wherein said fusion protein is made by  
30 genetic engineering techniques.
10. A pharmaceutical composition comprising a heparinase enzyme together with a pharmaceutically or a veterinarily acceptable carrier.
11. A pharmaceutical composition comprising fusion molecule  
35 comprising a ligand which binds to activated endothelium and a heparinase enzyme.

12. The pharmaceutical composition of claim 11, wherein said ligand binding domain is selected from the group consisting of cytokines, antibodies,  
5 integrins, and selectins.

13. The pharmaceutical composition of claim 11, wherein said ligand binding domain are fragments of said cytokines, antibodies, integrins, and selectins.

14. The pharmaceutical composition of claim 11, wherein said fragments  
10 are selected from the group consisting of cytokine receptor binding domains, Fab fragments, antibody variable regions, integrin I-domains, and selectin domains.

15. The pharmaceutical composition of either claims 10 or 11, wherein said carrier is selected from the group consisting of liposomes, lipospheres,  
15 proteosomes, microspheres, microcapsules, and biodegradable polymeric matrices.

16. The use of a heparinase enzyme in the preparation of a medicant for treatment of decreasing localized inflammatory responses in a patient's tissue.

17. The use of a heparinase enzyme in the preparation of a medicant comprising a fusion protein comprising a ligand which binds to activated  
20 endothelial cells and a heparinase enzyme for the treatment of decreasing localized inflammatory responses in a patient's tissue.

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### Abstract of the Invention

Heparinase enzymes can be used as a medical treatment to reduce localized  
5 inflammatory responses. Treatment of activated endothelium with heparinase  
inhibits leukocyte rolling, adhesion and extravasation. Most of the heparin and  
heparan sulfate on endothelial cell surfaces and in basement membranes is  
degraded by exposure to heparinase. In addition, immobilized chemokines, which  
10 are attached to heparin/heparan sulfate on activated endothelium are solubilized  
by heparinase digestion. Heparinase can be infused into the vascular system to  
inhibit accumulation of leukocytes in inflamed tissue and decrease damage  
resulting from localized inflammations. Targeting of heparinase to activated  
endothelium can be accomplished through localized administration and/or use of  
15 genetically engineered heparinase containing endothelium ligand-binding  
domains.

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#3

Applicant or Patentee: Bennett *et al.*  
Application Serial No.: 08/722,659  
Docket No.: 104385.140  
Filed or Issued: 27 September 1996  
For: Use of Heparinse To Decrease  
Inflammatory Responses



**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY  
STATUS (37 CFR 1.9 (f) AND 1.27 (c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:  
☒ an official of the small business concern empowered to act on behalf of the  
concern identified below:

NAME OF CONCERN	Ibex Technologies Inc.
ADDRESS OF CONCERN	5485 Paré
	Montréal, Québec
	Canada H4P 1P7

I hereby declare that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.801-805, and reproduced in 37 CFR 1.9 (d), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when, either directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled "Use of Heparinse to Decrease Inflammatory Responses," by inventor(s) Bennett *et al.*, which is described in

- ☐ the specification filed herewith  
☒ application serial no. 08/722,659, filed 27 September 1997  
☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).



\*\*\* TOTAL PAGE.003 \*\*\*

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME  
ADDRESS

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME  
ADDRESS

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

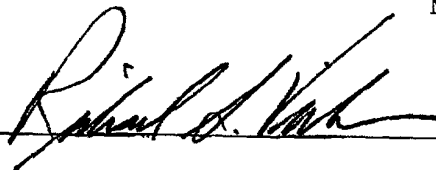
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING  
TITLE OF PERSON OTHER THAN OWNER  
ADDRESS OF PERSON SIGNING

Dr. Richard S. Vickers  
VP for Business Development  
5 Great Valley Parkway  
Malvern, PA 19355

SIGNATURE

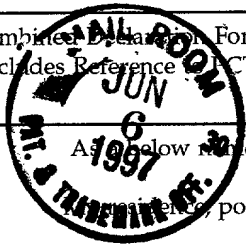


DATE

5-June-1997

#3

Combined Declaration For Patent Application and Power of Attorney (Includes Reference to PCT International Applications)	Attorney's Docket Number 104385.140
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As below named inventor, I hereby declare that:

My name, residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

USE OF HEPARINASE TO DECREASE INFLAMMATORY RESPONSES

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☒ was filed as United States application  
Serial No. 08/722,569  
on 27 September 1996  
and was amended  
on \_\_\_\_\_ (if applicable).
- ☐ was filed as PCT international application  
Number \_\_\_\_\_  
on \_\_\_\_\_  
and was amended under PCT Article 19  
on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate of any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119(a)-(d):				
Country (if PCT indicate "PCT")	Application Number	Date of Filing (day, month, year)	Priority Claimed Under 35 U.S.C. 119	
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

Combined Declaration For Patent Application And Power of Attorney (Continued) (Includes Reference to PCT International Applications)			Attorney's Docket Number  104385.140		
I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:					
PRIOR U.S. PROVISIONAL APPLICATION(S) FROM WHICH BENEFIT IS CLAIMED UNDER 35 U.S.C. § 119(e):					
Provisional Application Number			Filing Date		
60/004,622			29 September 1995		
I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
Prior U.S. Applications or PCT International Applications Designating The U.S. For Benefit Under 35 U.S.C. § 120:					
U.S. APPLICATIONS			STATUS (Check One)		
U.S. Application Number	U.S. Filing Date		Patented	Pending	Abandoned
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT Application No.	PCT Filing Date	U.S. Serial Number Assigned (if any)	Patented	Pending	Abandoned
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. <i>(List name and registration number)</i> <u>Hollie L. Baker, Reg. No. 31,321; Henry N. Wixon, Reg. No. 32,073; James B. Lampert, Reg. No. 24,564; Michael J. Bevilacqua, Reg. No. 31,091; Wayne M. Kennard, Reg. No. 30,271; Donald R. Steinberg, Reg. No. 37,241; Michael A. Diener, Reg. No. 37,122; Wayne A. Keown, Reg. No. 33,923; Ann-Louise Kerner, Ph.D., Reg. No. 33,523; Richard A. Goldenberg, Reg. No. 38,895, and Colleen Superko, Reg. No. 39,850.</u>					
Send Correspondence to:  Hollie L. Baker HALE and DORR LLP 1455 Pennsylvania Avenue, N.W. Washington, D.C. 20004-1008			Direct Telephone Calls to: <i>(name and telephone number)</i>  Hollie L. Baker (202) 942-8400		

Combined Declaration For Patent Application And Power of Attorney (Continued)  
(Includes Reference to PCT International Applications)

Attorney's Docket Number

104385.140

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of Inventor	Family Name <u>BENNETT</u>	First Given Name <u>D.</u>	Second Given Name <u>Clark</u>
Residence & Citizenship	City Montréal	State or Foreign Country CANADA	Country of Citizenship U.S.A.
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>D. Clark Bennett</u>			Date: <u>Apr 1 1997</u>
Full Name of Inventor	Family Name <u>CAUCHON</u>	First Given Name <u>Elizabeth</u>	Second Given Name
Residence & Citizenship	City Montréal	State or Foreign Country CANADA	Country of Citizenship CA
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>Elizabeth Cauchon</u>			Date: <u>March 27, 1997</u>
Full Name of Inventor	Family Name <u>FINK</u>	First Given Name <u>Dominique</u>	Second Given Name
Second Given Name	City Montréal	State or Foreign Country CANADA	Country of Citizenship CA
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>Dominique Fink</u>			Date: <u>MARCH 27<sup>th</sup> 1997</u>
Full Name of Inventor	Family Name <u>GROUX</u>	First Given Name <u>Brigette</u>	Second Given Name
Residence & Citizenship	City Montréal	State or Foreign Country CANADA	Country of Citizenship FR
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>[Signature]</u>			Date: <u>June 2, 1997</u>

Full Name of Inventor	Family Name <u>HSIA</u>	First Given Name <u>Ariane</u>	Second Given Name
Residence & Citizenship	City Montréal	State or Foreign Country CANADA	Country of Citizenship CA
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>Ariane Hsia</u>			Date: <u>May 26, 1997</u>
Full Name of Inventor:	Family Name <u>DANAGHER</u>	First Given Name <u>Pamela</u>	Second Given Name
Second Given Name	City Montréal	State or Foreign Country CANADA	Country of Citizenship CA
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>Pamela Danaher</u>			Date: <u>May 19, 1997</u>

Full Name of Inventor	Family Name <u>ZIMMERMANN</u> (7)	First Given Name <u>Joseph</u>	Second Given Name
Residence & Citizenship	City Montréal	State or Foreign Country CANADA	Country of Citizenship U.S.A.
Post Office Address	Post Office Address 5485 Paré	City Montréal	State & Zip Code/Country CANADA H4P 1P7
Signature of Inventor: <u>[Signature]</u>			Date: <u>May 22, 1997</u>

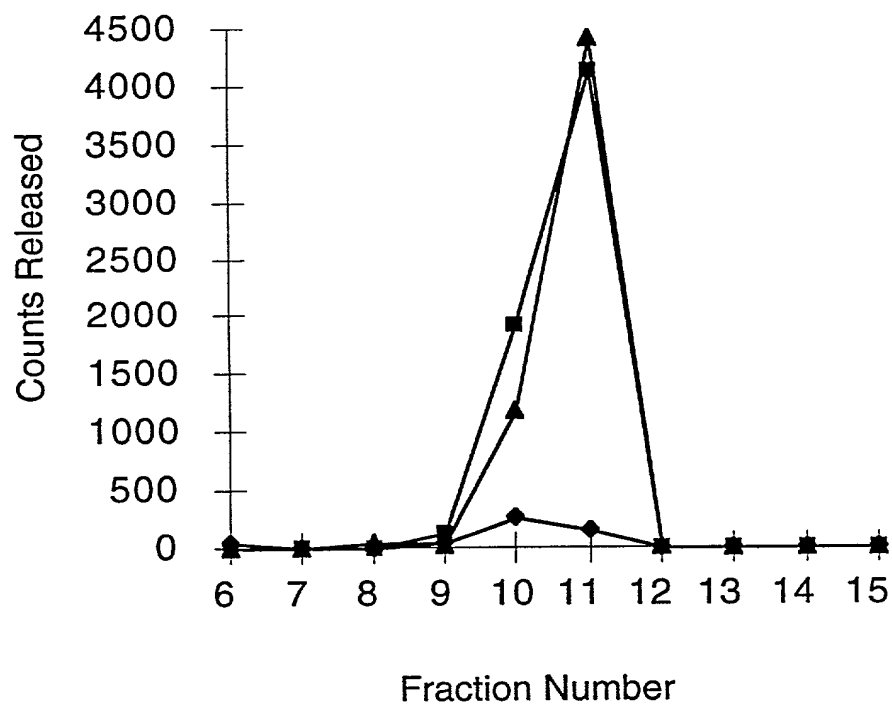


Figure 1

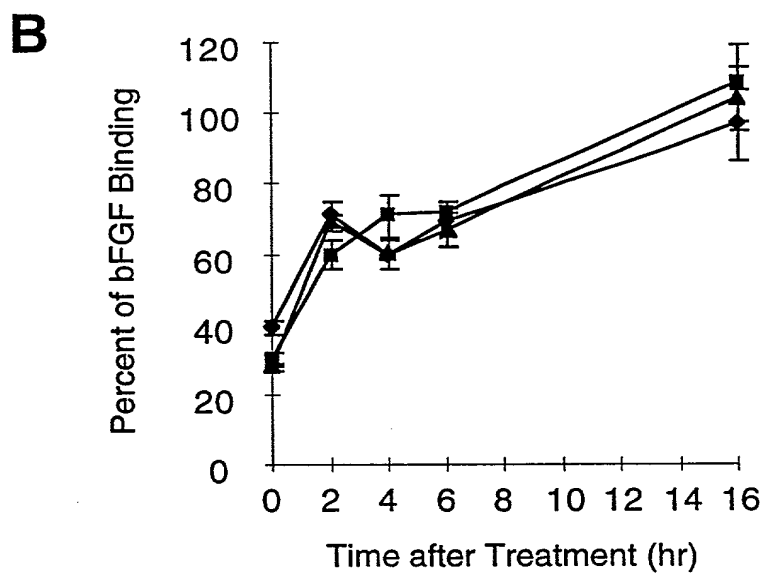
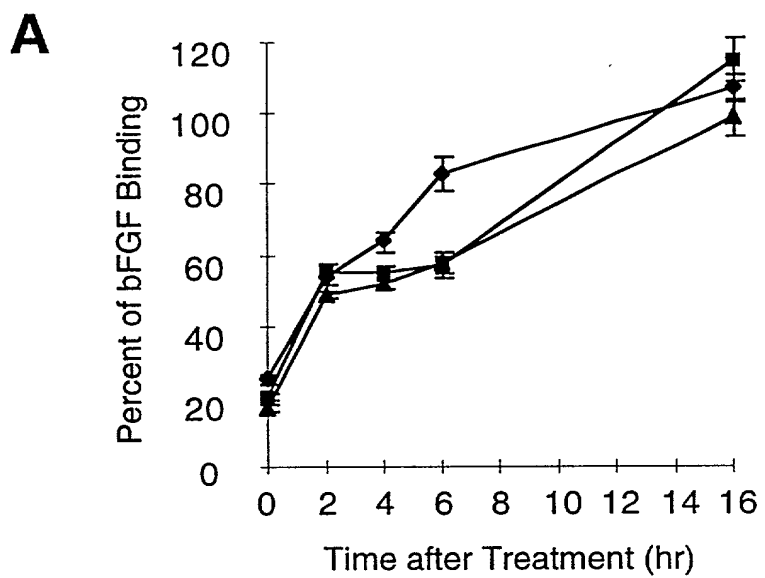
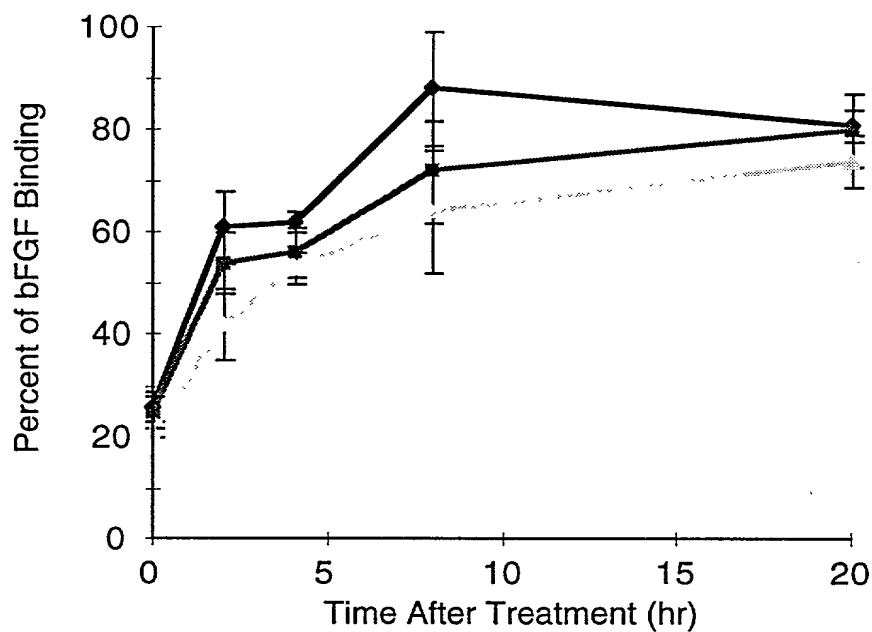


Figure 2

A.



B.

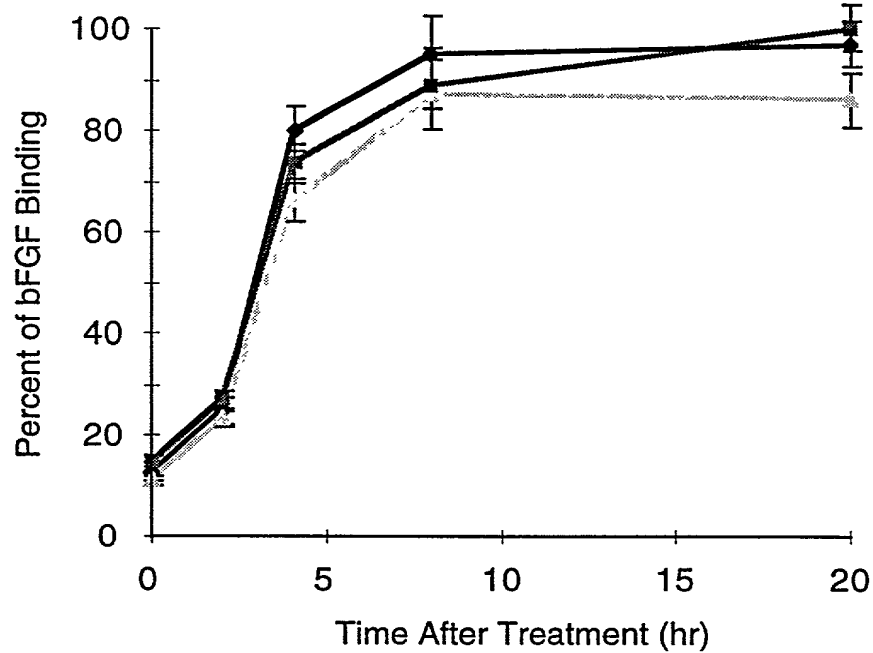


Figure 3



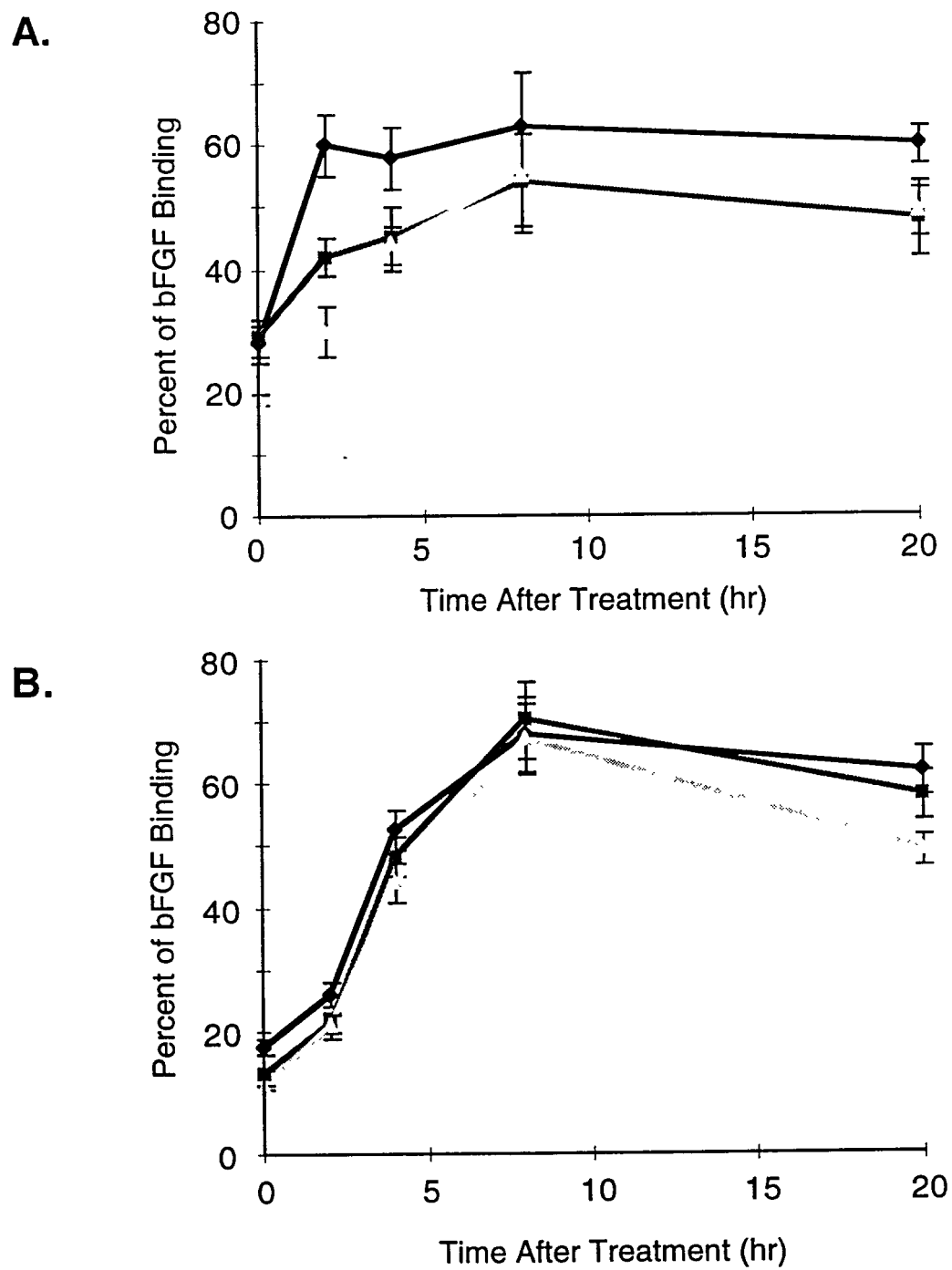


Figure 4

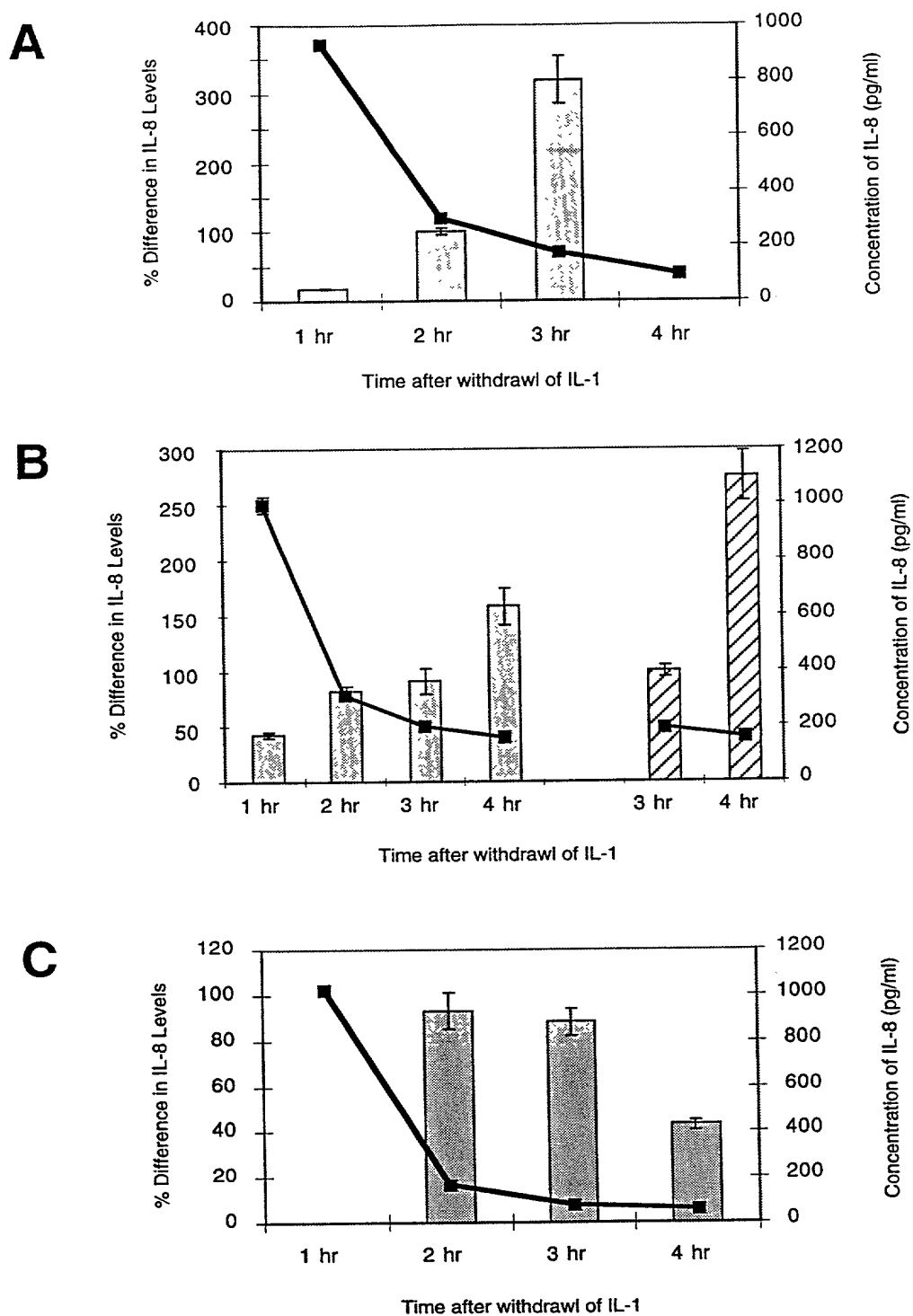


Figure 5

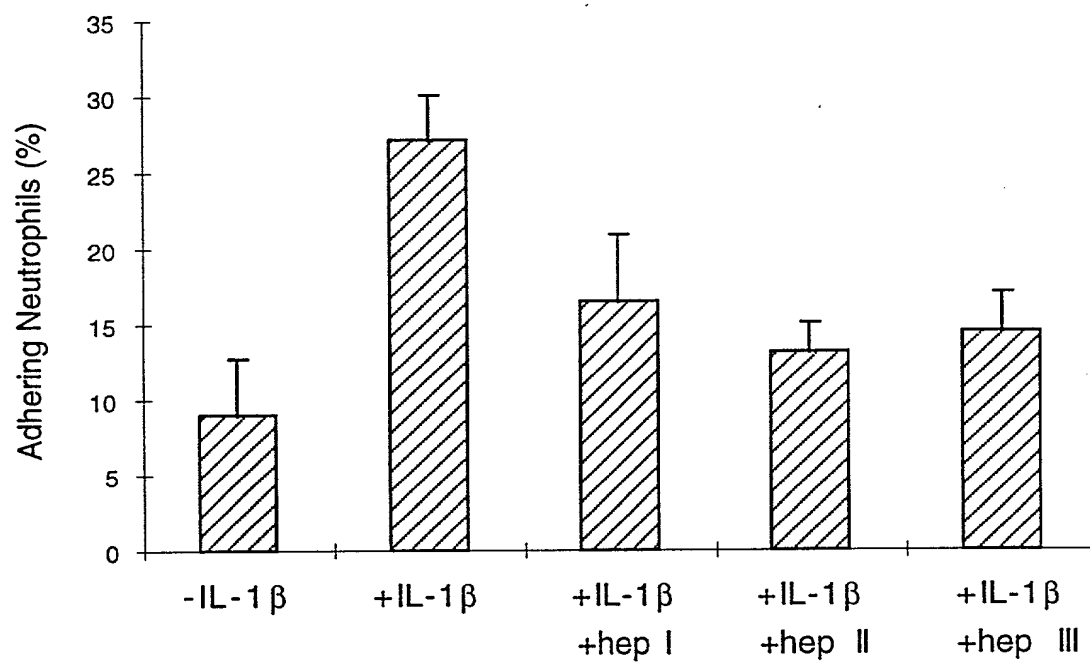


Figure 6

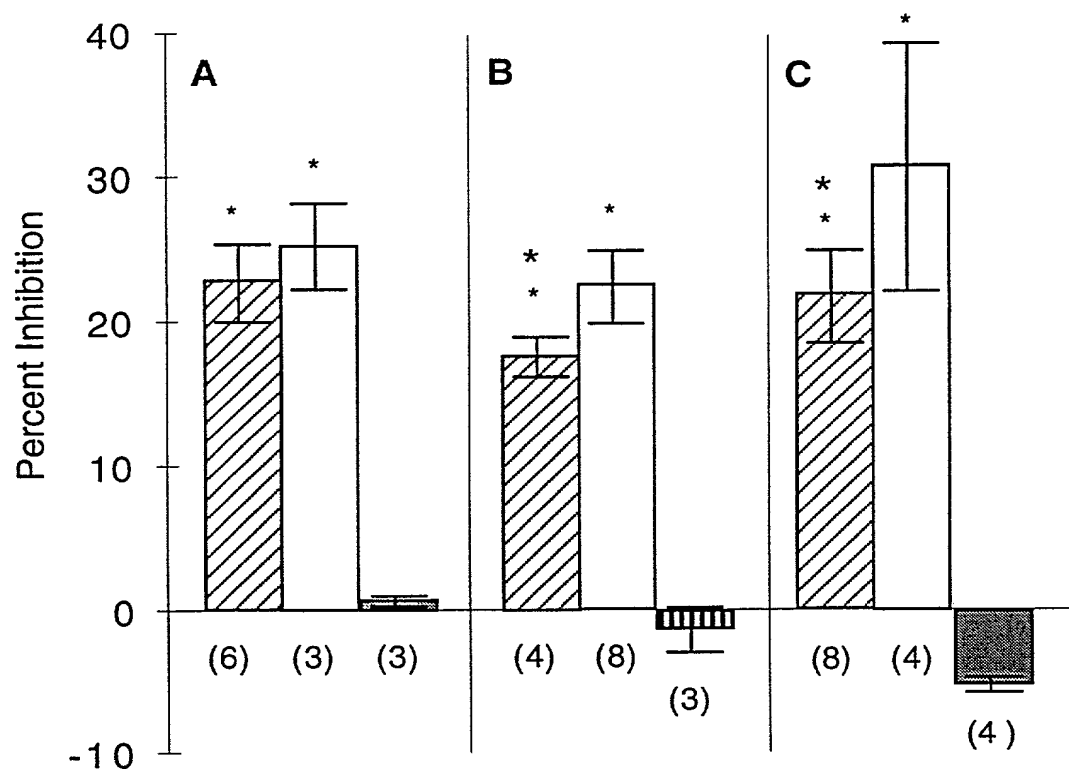


Figure 7

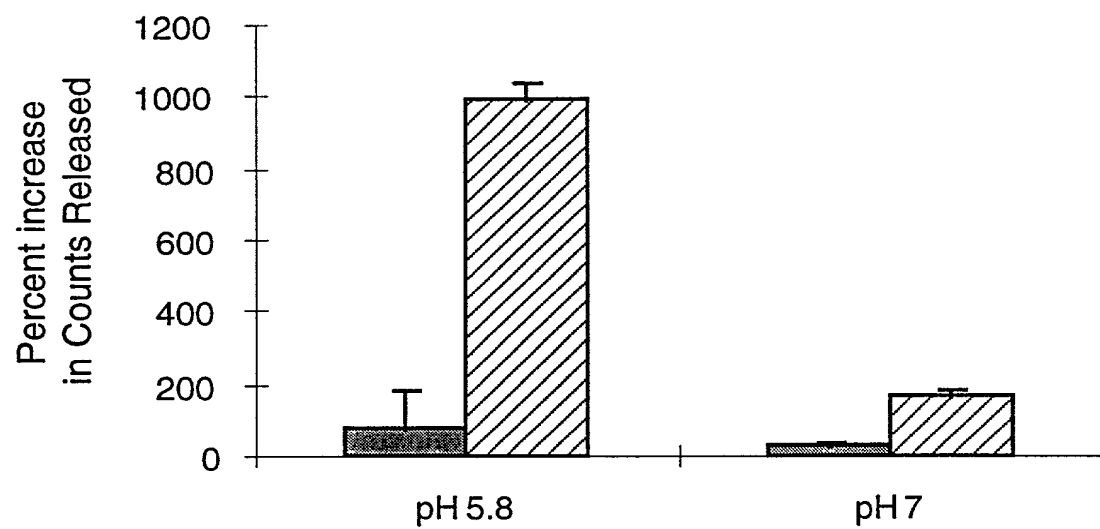


Figure 8

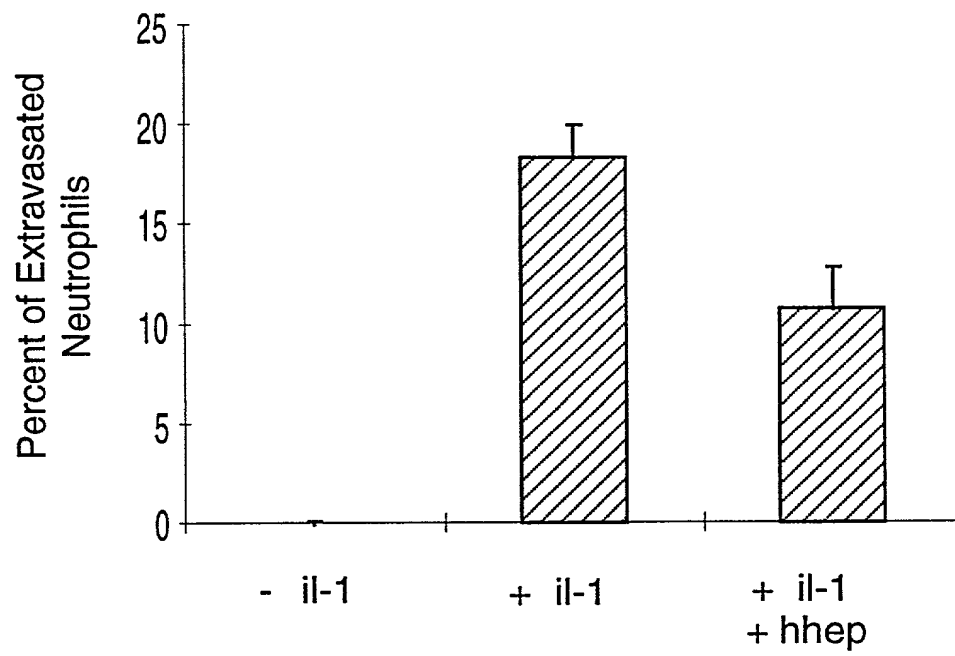


Figure 9

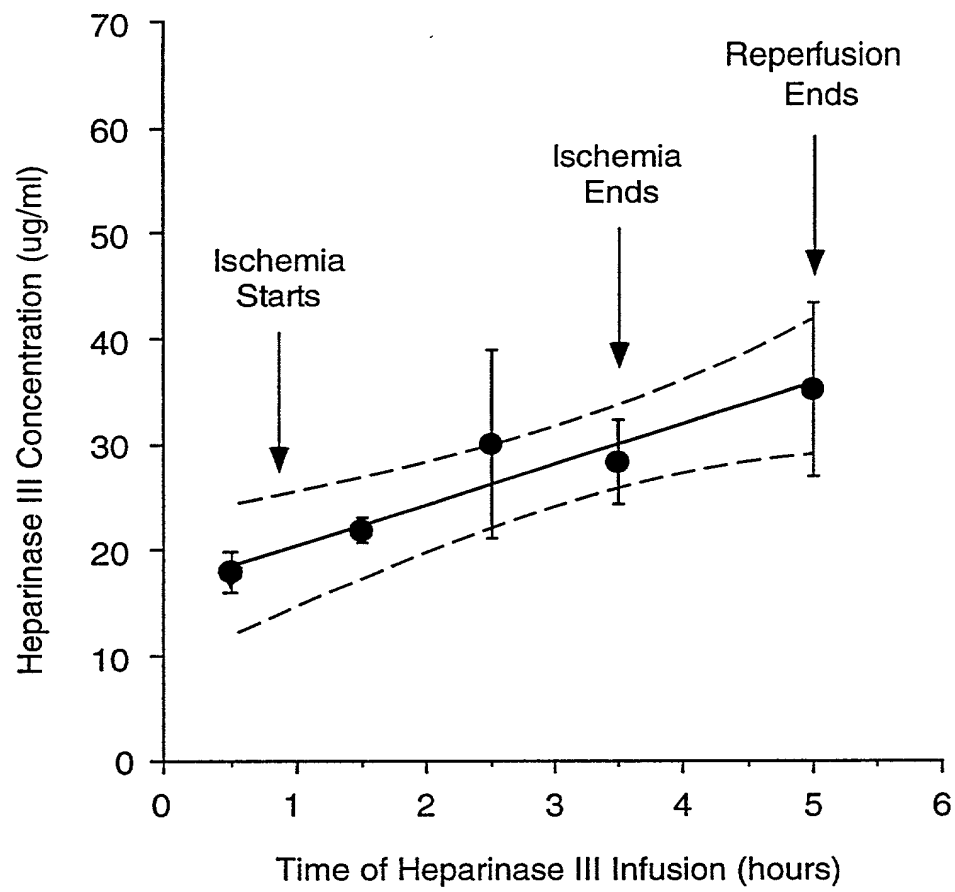


Figure 10

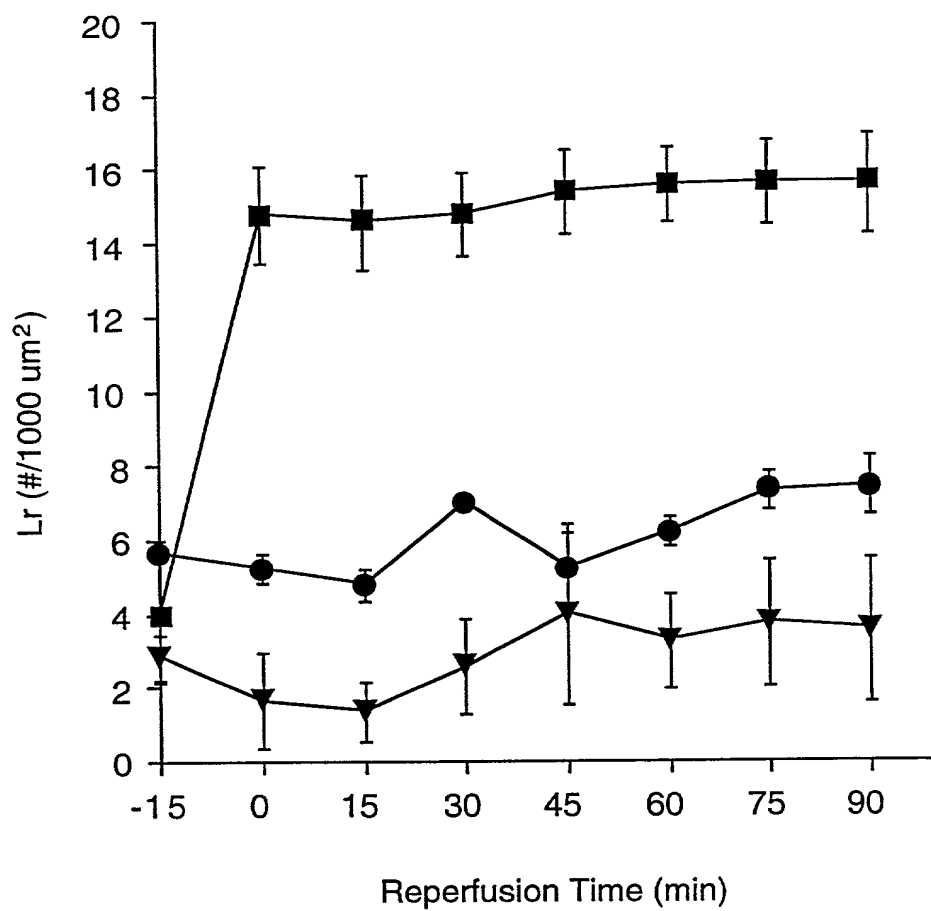


Figure 11



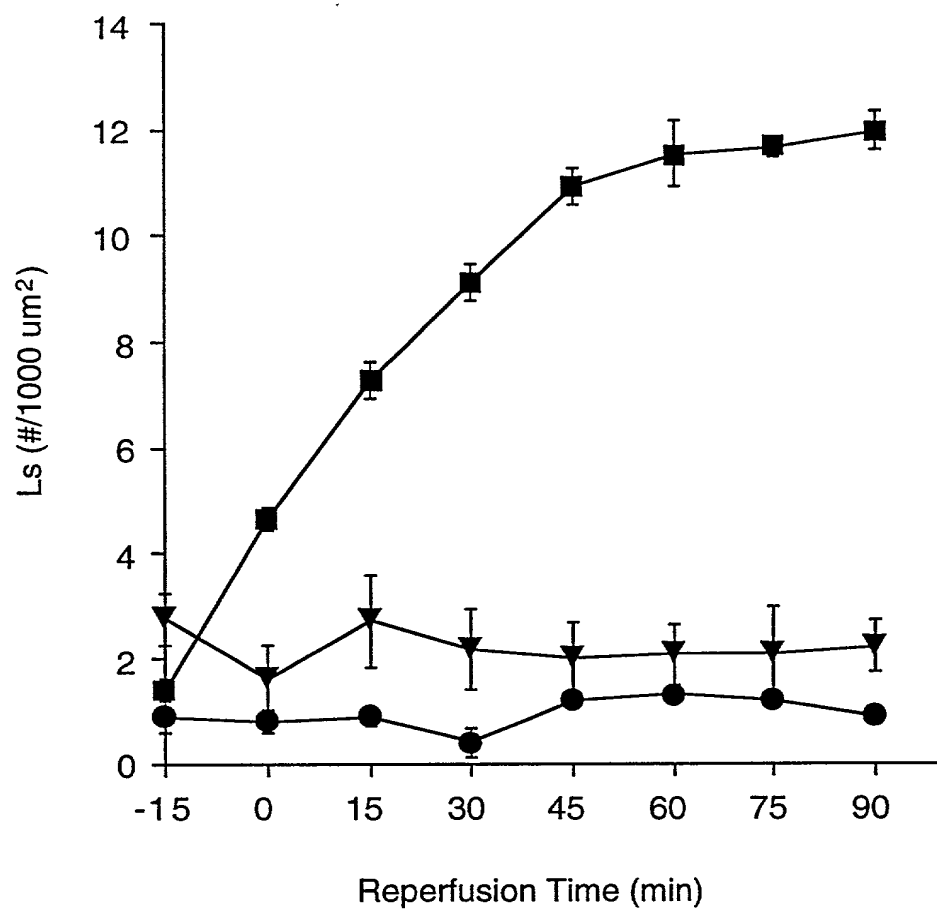


Figure 12

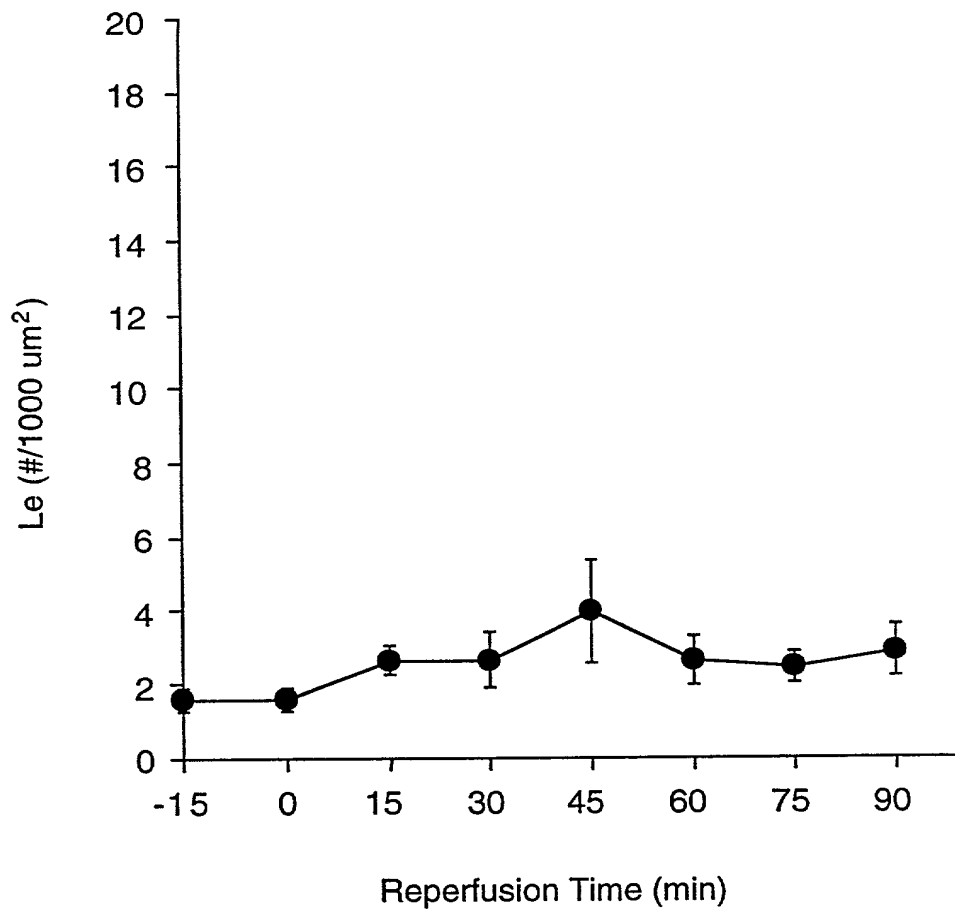


Figure 13

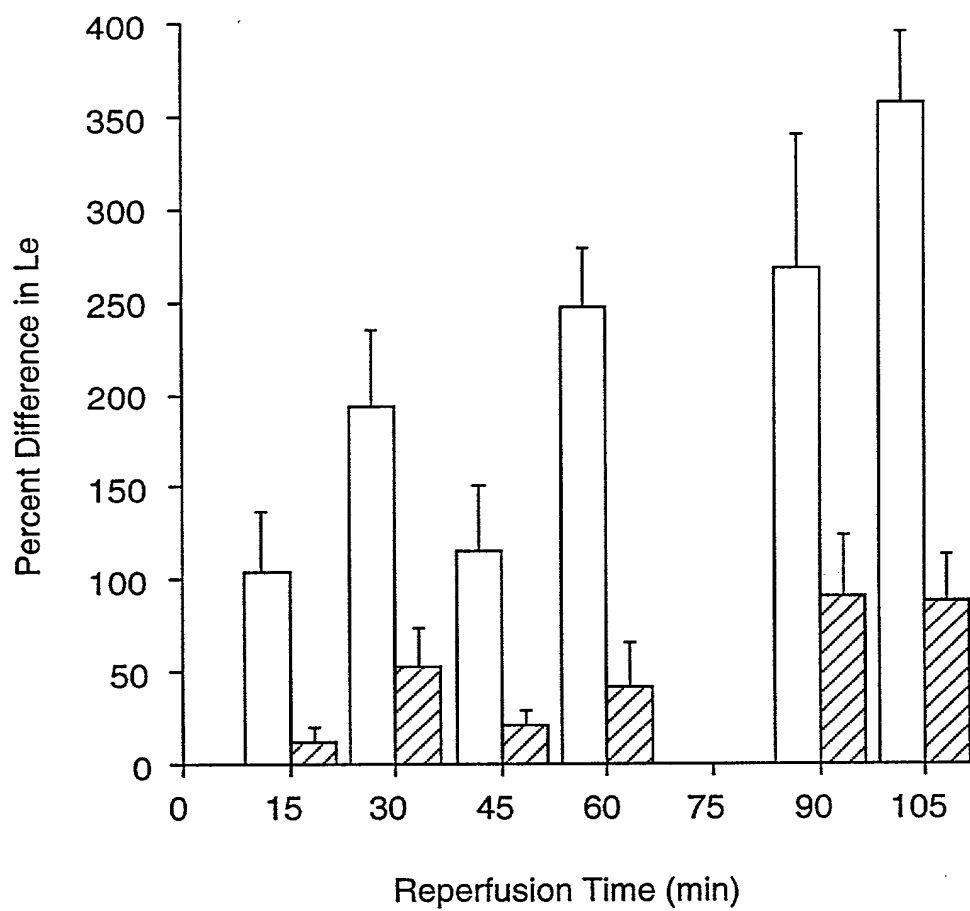


Figure 14

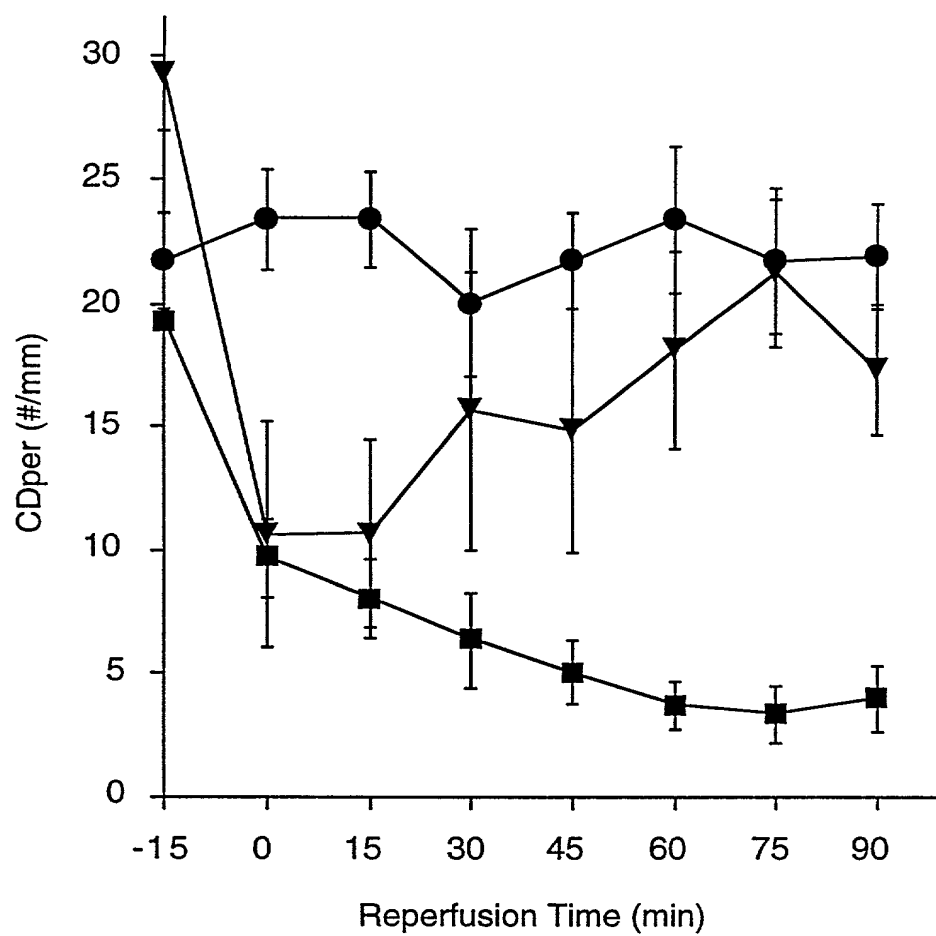


Figure 15

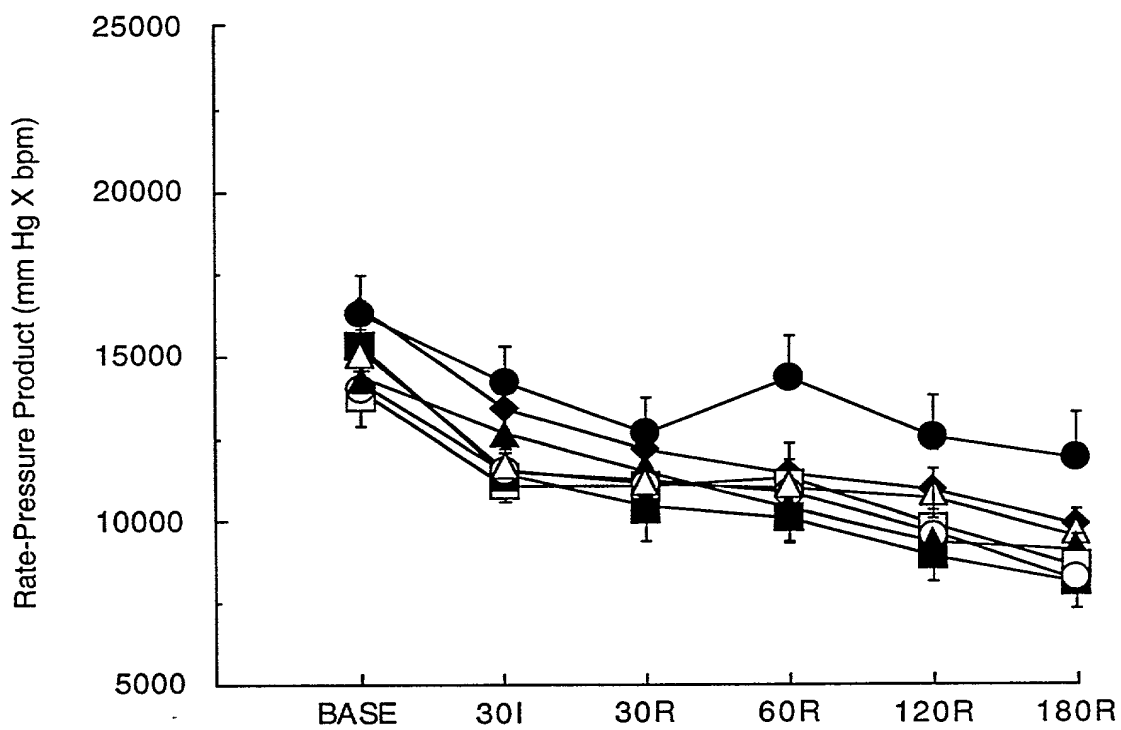
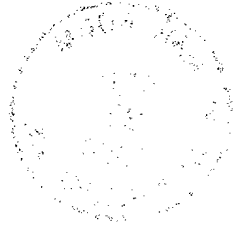


Figure 16

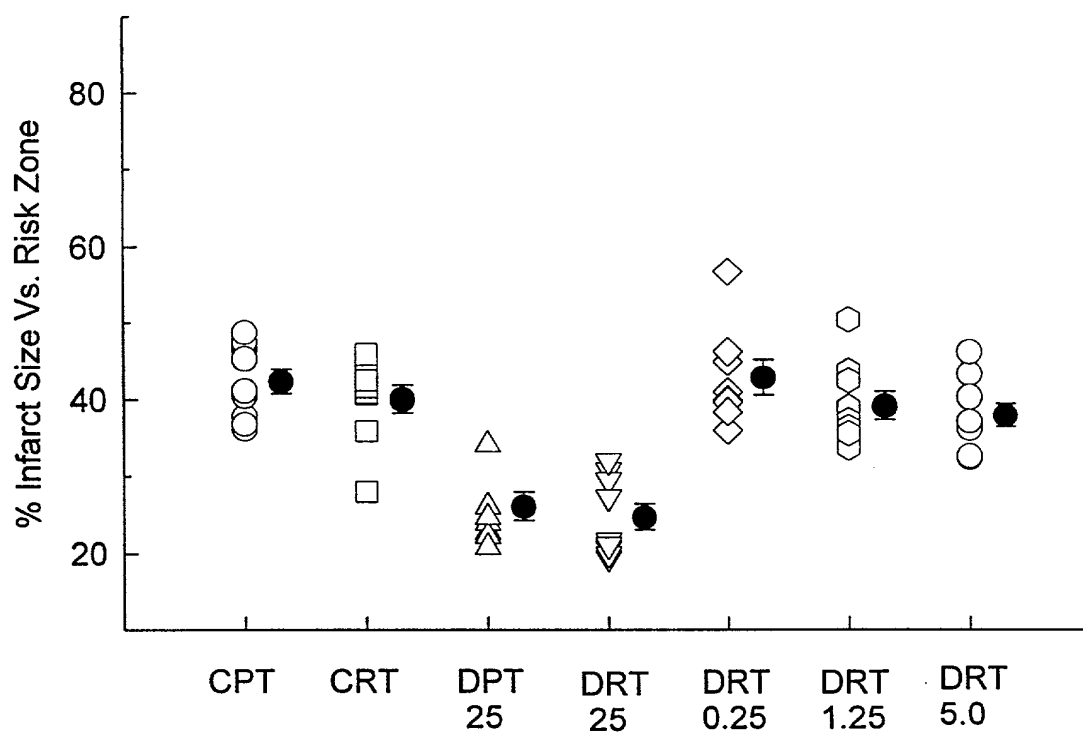


Figure 17

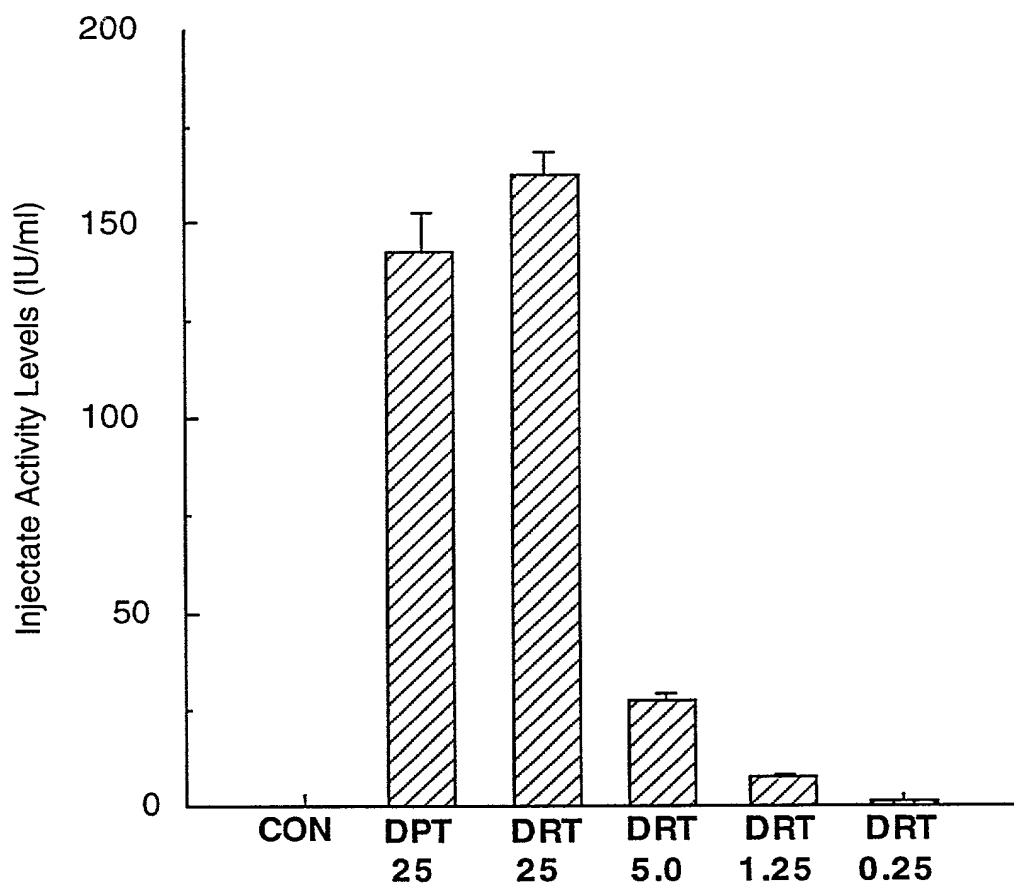


Figure 18

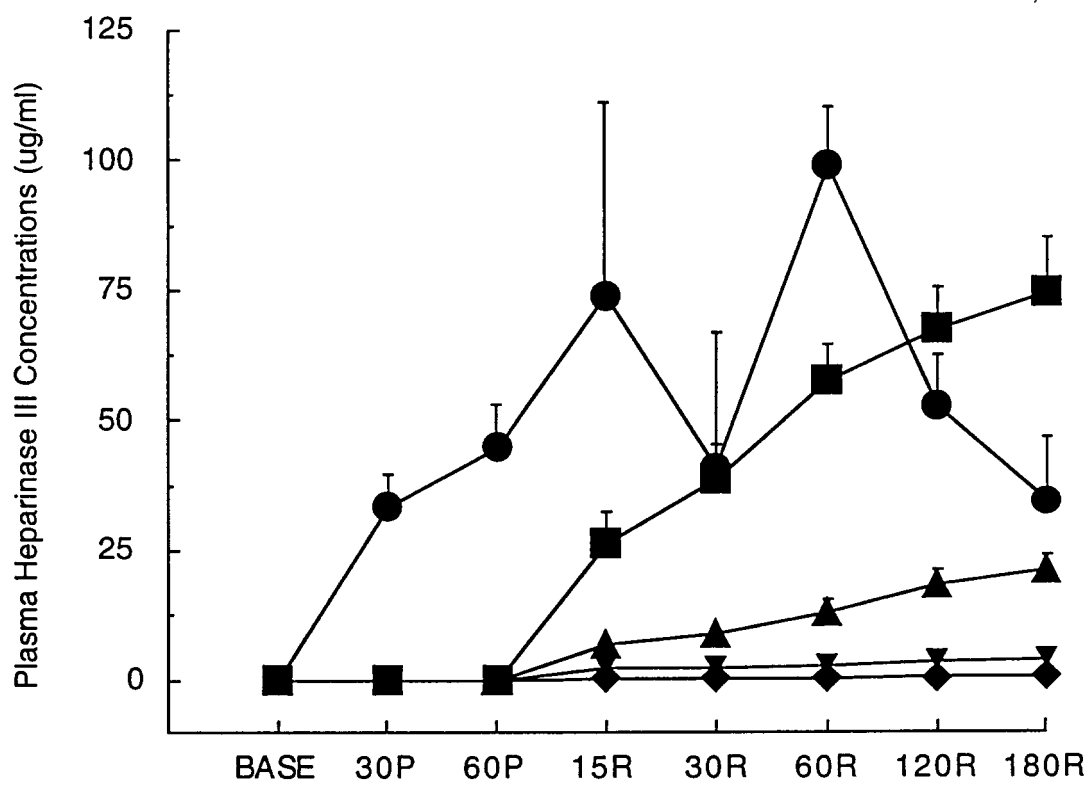


Figure 19